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PRINCIPAL INVESTIGATOR: Kenneth A. Iczkowski, M.D.

CONTRACTING ORGANIZATION: University of Colorado Health Science Center
Aurora, CO 80045-0508

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Kenneth A. Iczkowski, M.D.

Email: iczkoka@pathology.ufl.edu

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14. ABSTRACT Aim 1: Loss of CD44 standard and increased splice variant form CD44v7-10 facilitate prostate cancer (PC) invasion. First Sub-Aim: A manuscript was published (attached) on the role of Mitogen-activated protein kinase (MAPK) pathways and paracrine calcitonin, both of which dysregulate CD44. Second Sub-Aim: Metabolic labeling studies of CD44 total and CD44v7-10 protein were pursued over about a 6- month period, but the findings were not publishable.Aim 2: The use of adeno-associated virus for altering expression of CD44 prior to in vitro and in vivo studies was prohibitive owing to viral cytopathic effect. Instead, we used lentiviral or retroviral transfection methods in PC-3M cells, for stably altered expression. Confirmation of re-expression of CD44s as a Fusion protein (with luciferase) or Separate protein, or of RNAi knockdown of CD44v7-10, was achieved using qRT-PCR, western blot analysis, and IVIS visualization of luminescence after adding luciferin substrate, in a flask or mouse tumor. Cells re-expressing CD44s as a Separate protein had decreased growth, decreased Matrigel migration and invasion, decreased anchorageindependent colony formation, restoration of adhesion to hyaluronan (a benign feature), and apparently less tumor take and growth rate of subcutaneous xenografts in mice. Whether CD44s re-expression influences chemosensitivity to Docetaxel will require more experiments. Expression of CD44s as a Fusion protein was less definitive in assays. Effects of RNAi to CD44v7-10 are not clear yet.

15. SUBJECT TERMS
CD44, cell adhesion, alternate splicing, mitogen-activated protein kinase, calcitonin, pTracer plasmid

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INTRODUCTION:

CD44 is a transmembrane cell adhesion glycoprotein whose alternative splicing produces proteins that may function differently from each other. In 2008, we had three paper publications (1, 2, 3) on various aspects of CD44 function in prostate cancer. We now have a fourth in preparation (Appendix) concerning CD44 isoforms' roles in cancer invasion and growth.

In Aim 1, we used real-time RT-PCR and western blot analysis to show significant alterations on CD44 transcription, splicing, or both, caused by 1) exogenous calcitonin and 2) inhibition of certain components of the mitogen-activated protein (MAP) kinase pathway (1). In Aim 1, we also attempted pulse-chase experiments to study the turnover of CD44variant versus standard protein, but low abundance of these isoforms and technical complications were prohibitive.

For Aim 2, we decided to establish lentivector (rather than plasmid or AAV—as proposed earlier) prostate cancer cell transfectants to re-express CD44 standard isoform (anti-tumorigenic). We established other transfectants bearing RNA interference against pro-tumorigenic CD44 variant. These transfectants have been used in a series of in vitro assays as well as in mouse xenografts.

BODY:

Note on Timing and Personnel: My progress in the first year was limited because I had recently moved from the Gainesville, Florida, Veterans Affairs Medical Center and University of Florida to the University of Colorado, with a start date at work of March 23, 2007. Although funding became effective April 1, 2007, it took 3 months to get a Research Assistant.

Delays were due to the transfer of the award from my old institution (University of Florida) to my new one (University of Colorado Health Science Center); to the physical necessities of the move; and to the need to recruit by advertising; and to administrative start date constraints. **Mr. Eric Robbins**, MS (Microbiology) served from July, 2007, to July, 2008 as *Research Assistant*.

Using my start-up funds, **Dr. Emily Travanty**, Ph.D. (Microbiology) was hired as a *Fellow* September 1, 2007. Dr. Travanty had completed a 3-year fellowship in virology and remained with our laboratory for four months, at which time she left to take a Research Scientist position at another institution. In April, 2008, **Dr. Kui Yang**, Ph.D., was recruited as a *Fellow* and has now tapered to part time work followed by a leave of absence. **Dr. Alina Handorean** was a *Fellow* from July 2008 to March 2009. A 6-month cost-free extension was obtained from March 31 to September 30, 2009.

Aim 1: Mitogenic Pathway Effects on Splicing: Mr. Robbins, from July 1—December 1, 2008 accomplished Aim 1's first sub-Aim involving MAP kinase component which based on prior literature showed evidence of involvement in CD44 splicing (4) which we published (1).

Our CD44 variant primer/probe set (I brought from Florida) was suboptimal because the primers were too short and had a suboptimal G+C percent. The weeks from August to mid-September 2007 were taken up re-designing primer/probe sets for TaqMan for CD44 total (standard plus CD44v) and for CD44v7-10; with several experiments optimizing annealing temperatures and concentrations of probe and primer for the CD44s, CD44v,

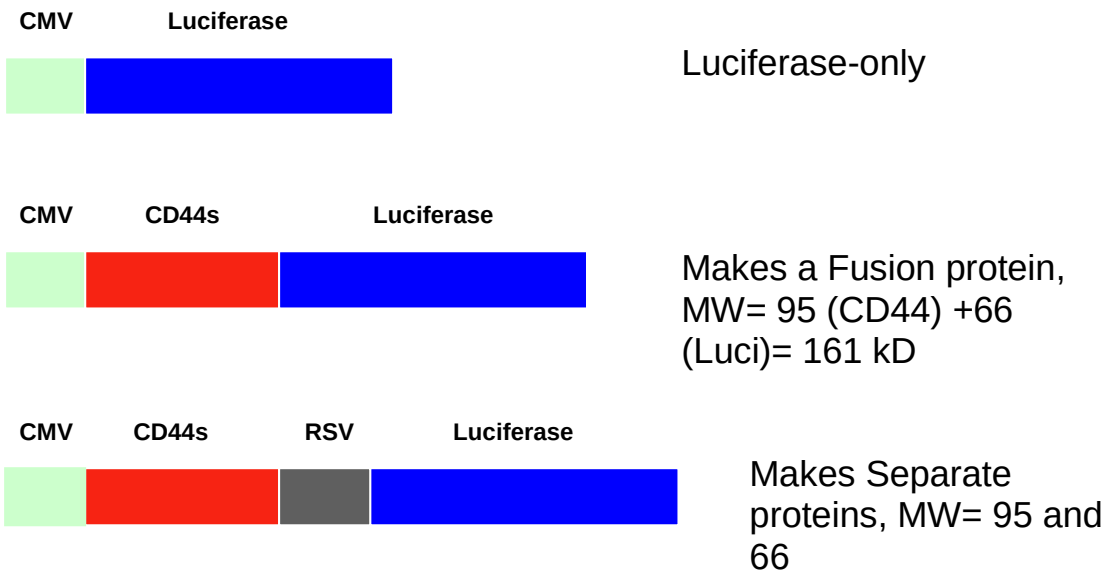
and the 18S ribosomal RNA control; and gaining proficiency making non-degraded RNA preparations. This involved some regular RT-PCR. RNA was isolated from treated or mock-treated cells in each experiment and examined by electrophoresis and optical densitometry.
The paper broadened this Aim to include not only testing of MEK and JNK inhibitors, but also p38 inhibitor, listed in the Alternatives section of the Aim. Further, involvement of the enzyme upstream to these three pathways, protein kinase A (PKA), was tested using its specific inhibitor H89.
Another expansion of Aim 1 was that we studied the effect of exogenous or endogenous calcitonin (CT) and its receptor (CTR). The rationale for this is my decade-long collaboration with Dr. Girish Shah (5-9) (Univ. of Louisiana) and his compelling demonstration of functional roles of CT and CTR in prostate cancer growth and invasion (10-20). As depicted in Fig. 7 of my proposal, we had proposed an action of CT-CTR axis on PKA. The response of CD44 total (mostly CD44s) and CD44v7-10 to exogenous CT was elucidated (1).

Metabolic labeling of proteins: This sub-Aim was pursued by Dr. Alina Handorean from September 2008 to February 2009. It was a metabolic study attempting to explain our observation that overexpression of CD44s caused an inhibition of CD44v (9). It involved radio-labeling the endogenous CD44v in cells in which re-expression of CD44s was (or was not) enforced.
The entire fall of 2008 and winter of 2009 were spent on this project, but there were two insurmountable problems. First, we did not have a reliable antibody for CD44v9. We had some of the v9 supernatant from ATCC's HB-258 left over from my previous lab in Florida from 2005-06, and it worked for the purpose of our MAP Kinase/calcitonin paper (1) but then ceased to work after several uses. Two purchases of the same hybridoma cell line from ATCC, in late 2008, yielded a supernatant that failed by dot blot and western blot analyses to detect CD44v9 in cells that should have been strongly positive for it, such as PC-3 and PC-3M.
The second problem was that the amount of CD44v7-10 protein was probably not abundant enough to immunoprecipitate.
A future alternative approach to examining this problem might use the luciferase vector that we developed for CD44 promoter (altered transcription) and, if we can make it work, the one for CD44v splicing. They might reveal a direct effect of CD44s overexpression on CD44v splicing.
Dr. Handorean's write-up of the project is appended (**Appendix 1**).

Aim 2: Approaches to this Aim using adeno-associated virus 2 (AAV2) approach were begun in 2007 by Dr. Travanty and Mr. Robbins. Subsequently, lentivirus (or other retrovirus) constructs were fully developed by Dr. Kui Yang in the summer of 2008. Using these constructs, we have performed a number of in vitro assays, and grown transfected cells (or controls) as tumors in 30 mice.
Last year's progress report described our efforts to use either a plasmid delivery system or AAV for transient or stable alteration of CD44, respectively. This will not be reiterated here. Basically, the studies using AAV were severely limited by cell viability. We realized by March 2008 that the viral construct was too cytotoxic.

After performing some experiments with our old, non-viral approach using PC-3 cells and transient pTracer plasmid, we switched to lentivirus vectors. Three constructs were made in Lentivector pLEX-MCS (Open Biosystems, Huntsville, AL), namely: Luciferase-only, Lenti-CD44s-luciferase, and Lenti-CD44s-RSV-luciferase (**Fig. 1**) as below. Lentivector contains the cytomegalovirus promoter, associated sequences, and puromycin resistance gene.

Figure. 1 CD44s luciferase constructs in Lentivector



Four shRNA constructs were made for knocking down CD44 variant 9 (of CD44v7-10). Three sequences were generated by Extractor 4 computer program, and the fourth was from our prior publication [7]. Retrovirus was produced from pSuper-RETRO (OligoEngine, Seattle) derived from pSuper. The RNAi construct, like the overexpression construct, contained a puromycin gene for selection. However, it lacked luciferase for detection. Thus, the only means of conforming knocked down CD44v7-10 were qRT-PCR (**Table 1**) or western blot (**Figs. 2-4**). Once these constructs were made, we confirmed the altered expression.

One way of demonstrating altered expression of luciferase, and by extension, of the accompanying protein, was to do western blot analysis directly for luciferase (**Fig. 2**)

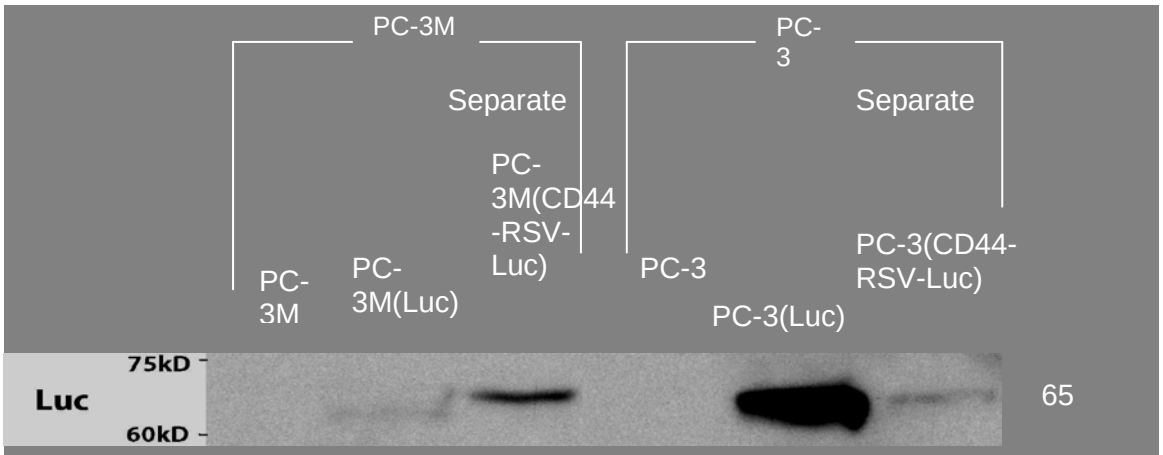


Fig. 2. Luciferase expressed separately from CD44s in PC-3M or PC-3

A second approach was to perform qRT-PCR with probe and primer sets specific for CD44 total directed against a CD44 standard exon (standard plus variant), or a set specific for CD44v9 (detects CD44v7-10).

Table 1. qRT-PCR confirmation of altered CD44 expression

Cells	**Normalized* CD44 total**	**Normalized*CD44 v7-10**
PC-3M Fusion	0.72	0.79
PC-3M Sep	6.58	1.0
1519 v7-10 RNAi	0.33	0.55
1522 v7-10 RNAi	0.58	0.096

*Normalized to untreated cells

As **Table 1** shows, CD44 total, most of which is CD44s, appeared more increased in PC-3M Separate cells than Fusion cells, but western blots showed both approaches were effective. RNAi against CD44v7-10 appears more effectively achieved by the 1522 construct than the 1519 one, and this is borne out in western blot analysis:

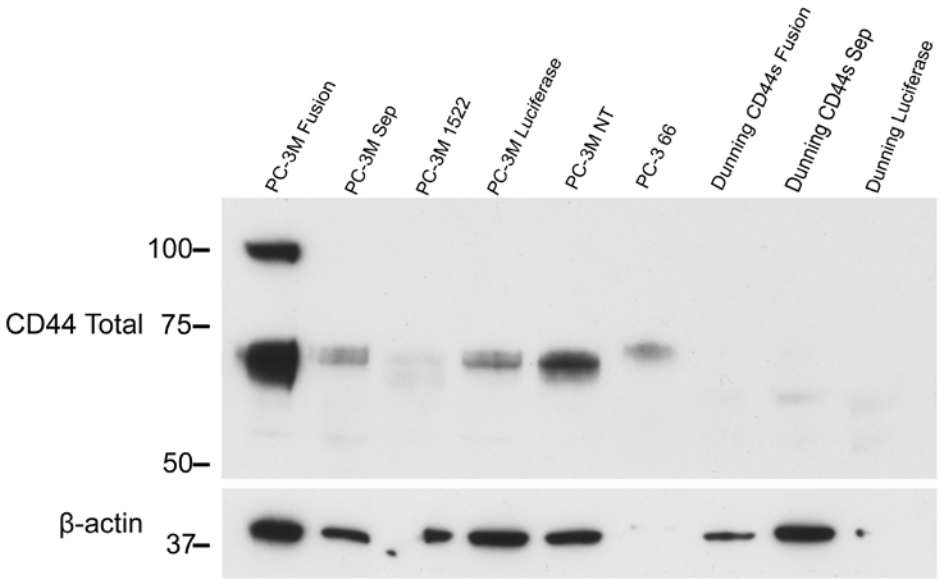


Fig. 3. Antibody to CD44 Total detects a predominant signal at 70 kD,

←

corresponding to CD44s. In the Fusion construct, CD44s-luciferase is noted at 105 kD, but there also is some CD44s as a separate protein.

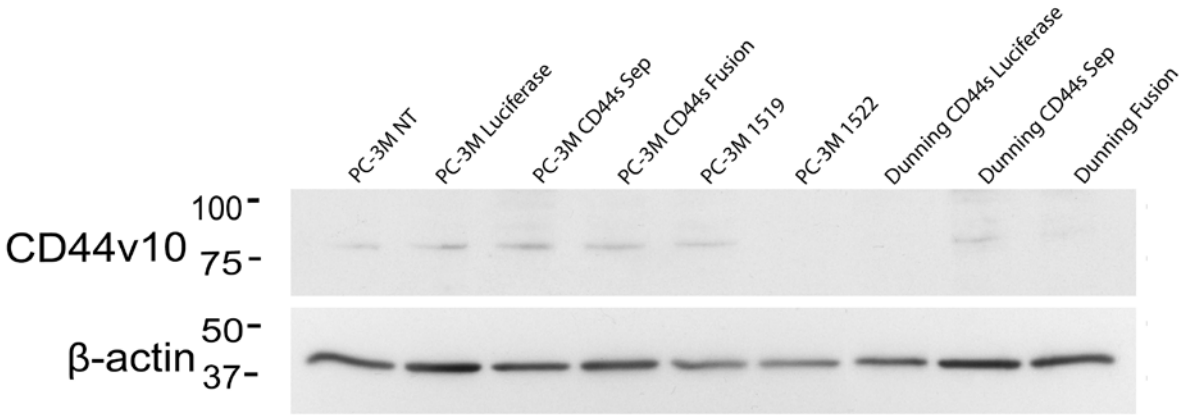


Fig. 4. To detect CD44v7-10, antibody to CD44v10 was used in lieu of the ineffective hybridoma supernatant for CD44v9. The detection of a predominant cleavage product at 80 kD rather than the >100kD uncleaved form, is consistent with our experience (1, 6-9). The re-expression of CD44s as a separate (Sep) or Fusion protein (lanes 3-4) was shown to have no effect on CD44v7-10. Two of our RNAi constructs, 1519 and 1522, were tested for knockdown of CD44v7-10. The knockdown was most successful in 1522, so 1522 was used for all future in vitro and in vivo assays.
RIGHT: The Dunning (rat) cells, containing the luciferase construct only, do not express CD44v7-10 detectable by the anti-human antibody we used.

Additionally, there were some important morphological differences observed between untreated (NT) PC-3M cells and the transfectants. The rounding up of the three types of transfectants suggests reverse epithelial-mesenchymal transition (EMT) (**Figure 5**). We are considering confirming this theory through western blot analysis for E-cadherin.

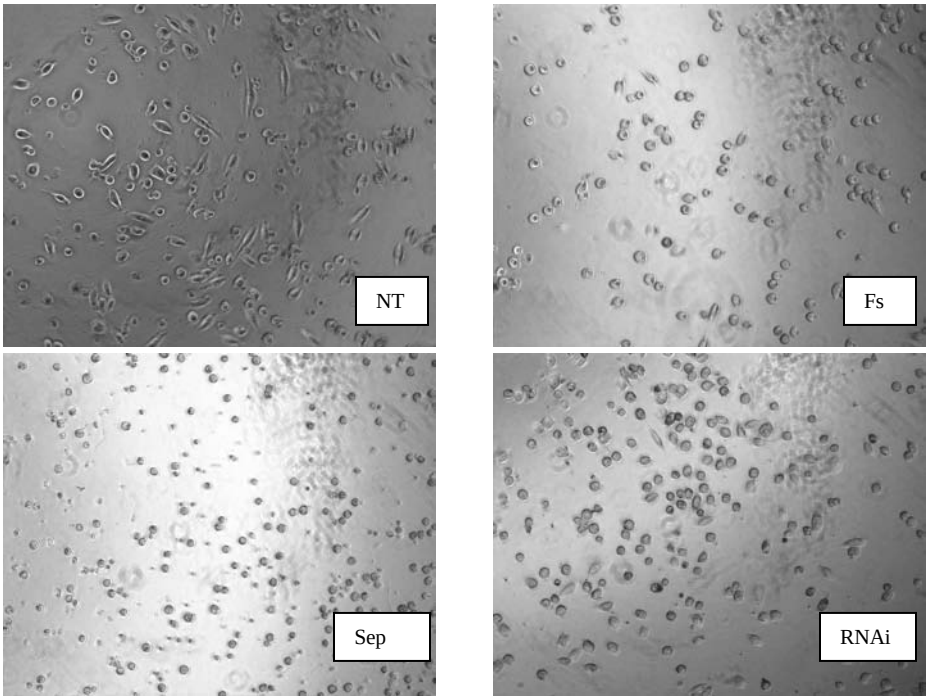


Fig. 5. The morphologies of PC-3M cells with no treatment (NT), CD44s-Fusion (Fs), CD44s-Separate (Sep) and cells with RNAi against CD44v7-10 (RNAi) are shown.

The constructs were then tested with a series of *in vitro* assays.

1. Anchorage-Dependent Growth (**Fig. 6**).

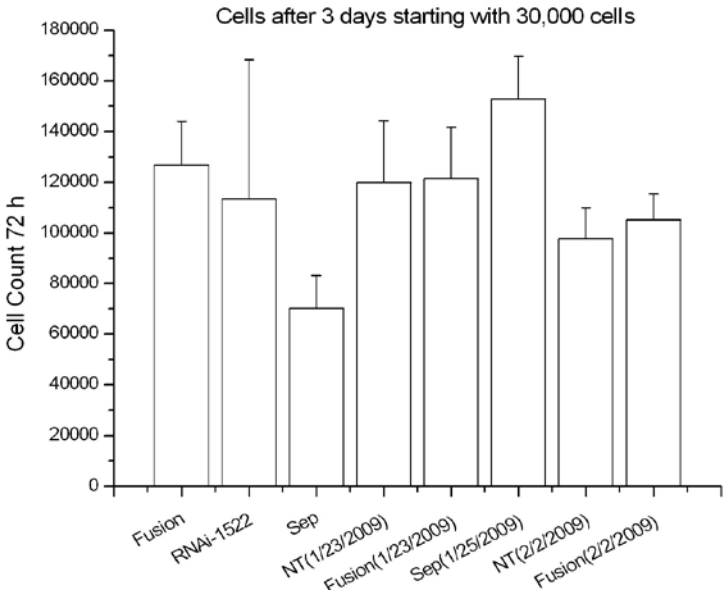
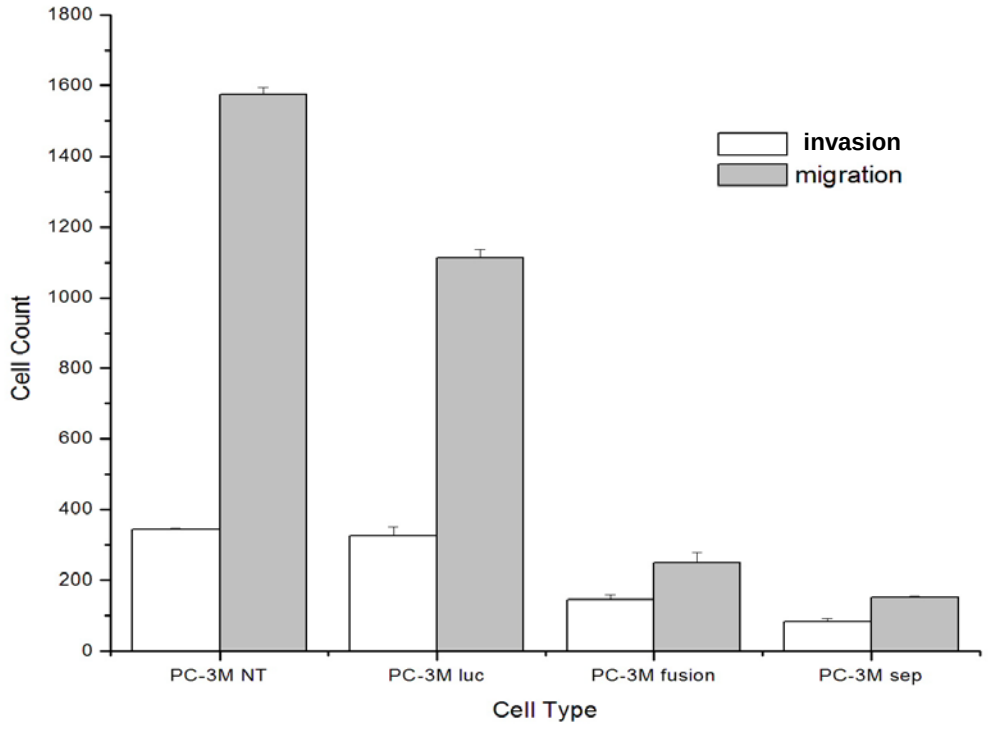


Figure 6. After plating 30,000 PC-3M cells and 72 hours' growth, the cells were thoroughly trypsinized and counted. There is evidence that the PC-3M cells with expression of CD44s as a separate protein have decreased growth.

1. Matrigel Invasion Assay as per Omara-Opyene et al. (7) was done, followed by western blot analysis of the invaded versus non-invaded cells, compared with untreated PC-3M cells (**Fig. 7**).

Figure 7. Assay of PC-3M cells invading Matrigel insert or migrating through control insert



2. MTS Proliferation assays after transfection. The PC-3M cells were transfected with either the CD44v7-10 RNAi construct, or the re-expression construct for CD44s as a fusion protein with luciferase. MTS (Sigma-Aldrich or ATCC) colorimetric survival assay was done similar to Thomas et al. (17) in prostate cancer. Triplicate wells of 80,000 cells/well were plated out on a 96-well plate 24 h prior to the experiment. Cells were incubated 2 h at 37 C followed by cell lysis with 1 mL lysis buffer with 20% SDS for 6 h. Cell lysates examined for

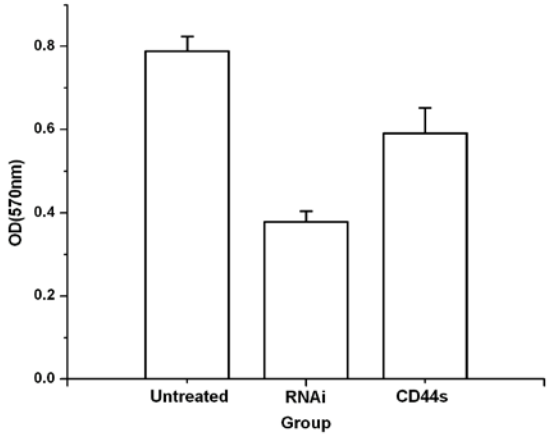


Figure 8. MTS assays. RNAi against CD44 v7-10 brought about 50% decrease in proliferation, while overexpression of CD44s—fusion protein caused a 25% decrease.

optical absorbance at 595 nm. Viable cells = optical absorbance treated/optical absorbance control. ANOVA with 2-sided test was performed.
MTS demonstrated that both approaches decreased proliferation (**Fig. 8**).

3. Anchorage-independent growth by soft agar colony formation assays. In the manner of Chien et al. (11) and Thomas et al. (17), we assessed the number of colonies formed after plating 5,000 cells per 2.5 cm dish and waiting 2 weeks. The only cells appearing to form significantly fewer colonies were the PC-3 (not PC-3M) cells expressing CD44s as a separate protein (**Fig. 9**). More repeat experiments are needed.

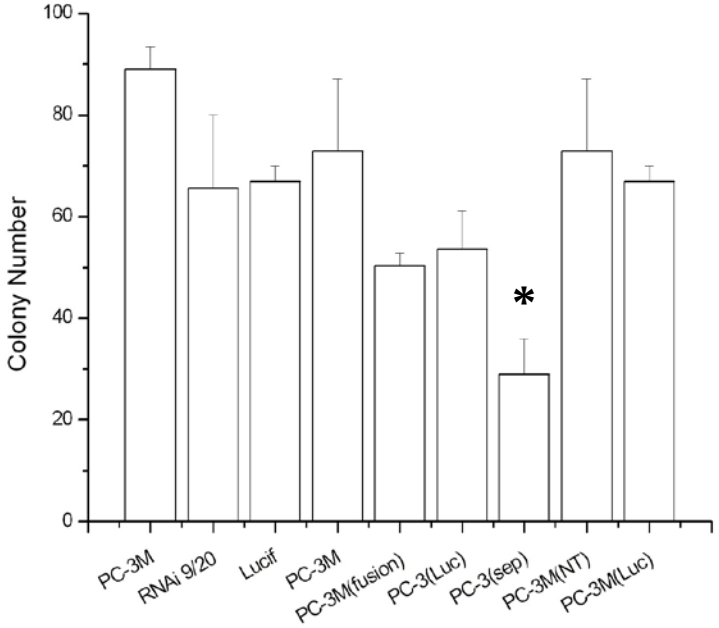
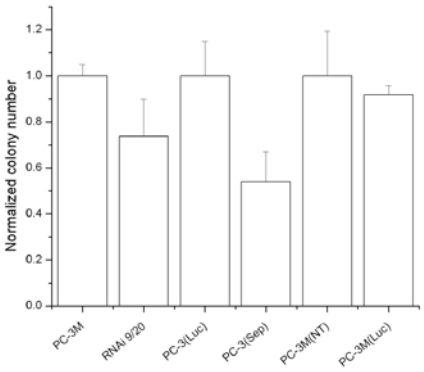


Figure 9. Colony formation in soft agar after 14 days. In PC-3 cells, colonies were decreased compared to luciferase-only. This needs to be repeated for PC-3M.



3. Chemosensitivity assay against Docetaxel. PC-3M cells were plated overnight and treated for 48 hours with a spectrum of doses of Docetaxel, the most commonly used chemotherapy drug for prostate cancer patients. The MTS assay was performed to assess proliferation as in Zhang et al. (21). **Figure 10a** shows the proliferation rate of cell lines treated with no Docetaxel (dose 0) and 5--100 nM. Because the proliferation curves were not equal at the 0 dose point, we normalized the results of all other doses to the proliferation at the 0 dose, allowing comparison of doses of 5--100 nM (**Figure 10b**).

The main problem with these results is that the luciferase-only PC-3M cells are showing greatly inhibited proliferation compared to the untreated (NT) PC-3M cells. This result may have reflected suboptimal cell status of the luciferase-only cells. Obviously, this experiment bears repeating, and we are doing so presently.

The chemosensitivity assays were also attempted with the Dunning cells, but the Dunning cells were resistant to Docetaxel. However, given the above-stated problems with the Dunning cells as a model for altering CD44 expression, that result is irrelevant.

Figure 10a. Response of PC-3M cells with no treatment (NT) or expressing CD44s as a Fusion or Separate protein, or with RNAi (1522) against CD44v7-10, or with luciferase vector only. Not normalized to account for different proliferation rates at the 0 dose.

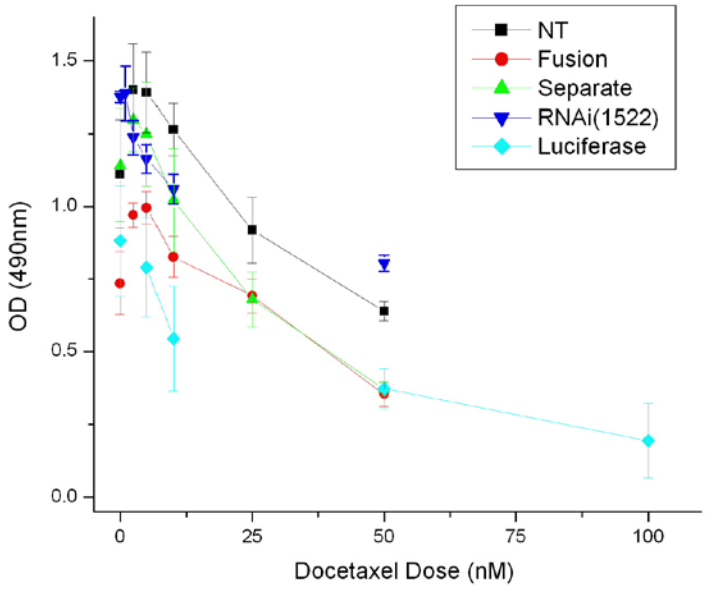
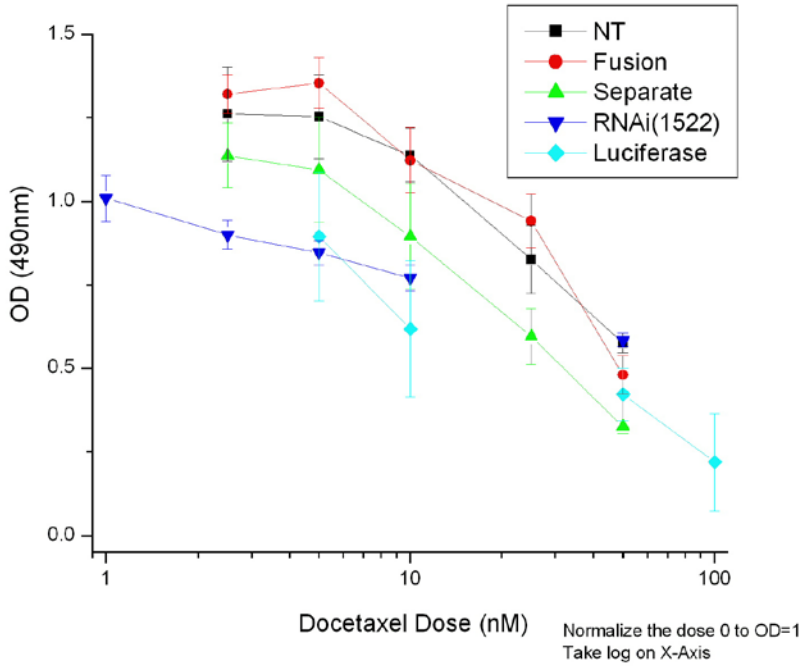


Figure 10b. Response of PC-3M cells with no treatment (NT) or expressing CD44s as a Fusion or Separate protein, or with RNAi (1522) against CD44v7-10, or with luciferase vector only. Normalized according to the 0 dose.



6. In vivo growth assays in mice. After these tests, either pretreated PC-3 cells or Dunning rat prostate cells, were grown subcutaneously in nude mice. The cells were imaged with an IVIS (In Vivo Imaging System, Xenogen Corp., Hopkinton, MA). No metastatic tumor was found from the PC-3M cells (as expected) nor from the Dunning cells (in which metastases were expected). We have used 30 mice to date, as detailed in in Table 2. The results for the PC-3M cell transfectants are shown in **Figure 11**, and those for the Dunning cell transfectants in **Figure 12**.

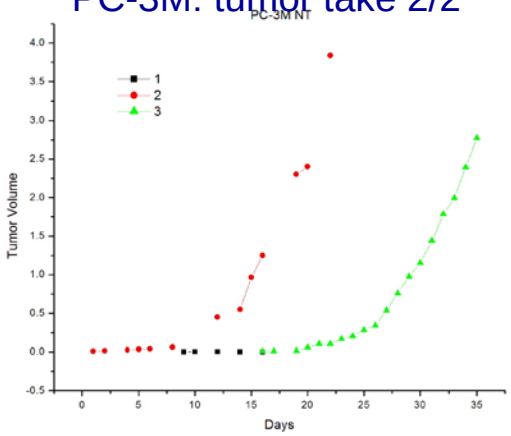
Table 2. Use of mice for Aim 2 of the project

Mouse	Date	Cells used	Cumulative total
1	8/8/08	PC-3M	1
2	8/8/08	PC-3M scrambled: no growth	1
2	9/19/08	PC-3M fusion: no growth	1
2	10/13/08	PC-3M: no growth	Don’t count
3	10/2/08	PC-3M fusion	2
4	10/2/08	PC-3M	2
5	11/11/08	PC-3M	3
6	11/11/08	PC-3M separate	1
7	11/21/08	PC-3M fusion	3
7	12/16/08	PC-3M CD44v-RNAi: no growth	1
8	12/24/08	PC-3M CD44v-RNAi	2
9	12/24/08	PC-3M luciferase	1
10	12/24/08	PC-3M fusion: no growth	4
11	2/4/09	PC-3M fusion: no growth	5
11	3/9/09	PC-3M separate	2
12	2/25/09	Dunning-luciferase	1
13	2/25/09	Dunning-luciferase	2
14	2/25/09	Dunning-fusion	1
15	2/25/09	Dunning-fusion	2
16	3/11/09	Dunning-separate	1
17	3/24/09	Dunning-separate	2
18	3/24/09	Dunning-separate	3
19	3/24/09	Dunning-luciferase	3
20	3/24/09	Dunning-luciferase	4
21	4/9/09	PC-3M	4
22	4/9/09	PC-3M luciferase	2
23	4/9/09	PC-3M fusion	6
24	4/15/09	PC-3M separate	3
25	4/9/09	PC-3M separate	4
26	4/27/09	PC-3M CD44v-RNAi	3
27	4/27/09	PC-3M CD44v-RNAi	4
28	4/27/09	PC-3M CD44v-RNAi	5
29	4/27/09	PC-3M separate	5
30	4/27/09	PC-3M	5

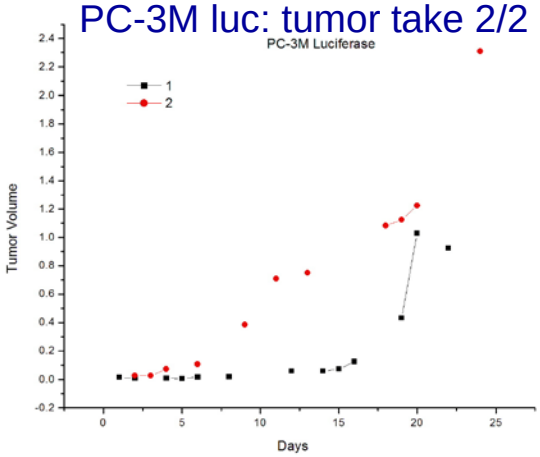
Figure 11.

PC-3M CELLS GROWTH IN MICE

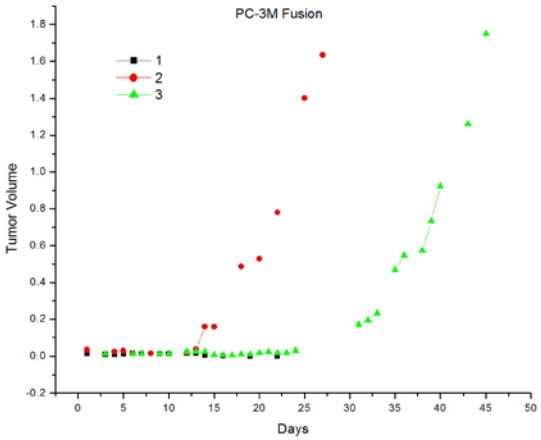
PC-3M: tumor take 2/2



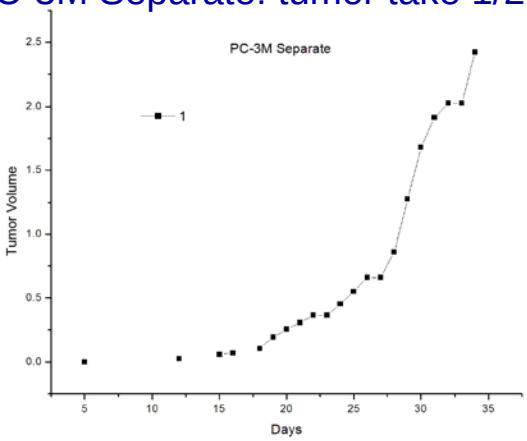
PC-3M luc: tumor take 2/2



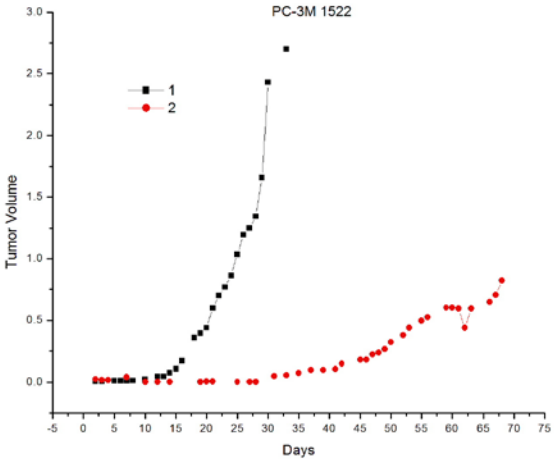
PC-3M Fusion: tumor take 2/5



PC-3M Separate: tumor take 1/2



CD44v7-10 RNAi: tumor take 2/2

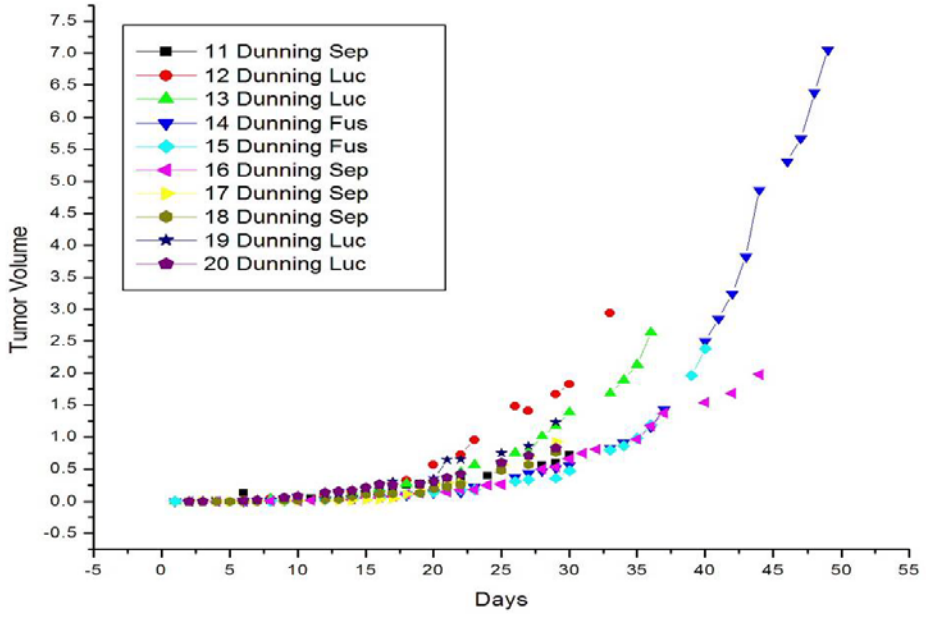


Several conclusions are apparent:

1) In PC-3M untreated cells or those with luciferase only, the tumor 'take' was 100%, while with PC-3M-CD44s fusion or separate cells, the tumor take was ≤50%.
2) The growth rate in the fusion or separate cells has been generally less than in untreated or luciferase cells, as you will note that the Y-axis is in smaller intervals.
3) There are still some important disparities in growth rates <u>within</u> a given cell transfectant type, but experiments are now in progress toward the goal of n≥5 mice for each group.

We also performed experiments using Dunning rat cells (**Figure 12**). We had been sufficiently convinced on the basis of just a weak-to-moderate luciferase signal emitted by transfected cells that we had achieved expression of the desired sequences. However, after repeating several western blot analyses we came to the conclusion that we failed to alter CD44s or CD44v7-10 noticeably in the transfected cells. Tumors were grown prior to our finding that we had not achieved good CD44s re-expression or CD44v7-10 knockdown. There is a minimally slower growth rate in the CD44s Fusion (Fus) and CD44s Separate (Sep) cells but this is probably not significant. We have discontinued using Dunning cells for both in vitro and in vivo experiments and are concentrating on just the PC-3M cells for now.

Figure 12. DUNNING RAT CELLS GROWTH IN MICE



Growth rates were also studied by in vivo growth of the Dunning rat cells, but that was before repeated western blot analysis showed that the Dunning CD44s re-expression cells as well as Dunning CD44v7-10 RNAi cells were similar to untreated Dunning cells.

In conclusion, the **Table** below summarizes our observations regarding the transfected PC-3M cells.

	CD44s-Sep	**CD44s-Fus**	**RNAi to CD44v7-10**
Growth	↓		
Matrigel migration & invasion	↓	↓	
Soft agar colony formation	↓		
Hyaluronan adhesion	↑		
Chemosensitivity to Docetaxel	Need repeat		
Xenograft tumor take and growth	↓	↓	?

Our project is still in progress and over the coming 2 months, we hope to finish several experiments to supplement the above results and provide sufficient data for manuscript submission.

KEY RESEARCH ACCOMPLISHMENTS:

1. Completion of sub-Aim 1 under Aim 1; publication of manuscript (1).

2. Based on the discovery that miR-373 and miR-520c had important interactions with CD44 in breast cancer (22), we investigated their effects in prostate cancer (2). We used the *luciferase promoter construct* to test further the effects of these miRs and found them to act somewhat differently in prostate cancer.

3. Because of the interest at Univ. of Colorado in the nutritional chemoprevention of prostate cancer by the anti-growth compound Silibinin, we collaborated with Dr. Rajesh Agarwal, of the Pharmacology Department, and found an inhibitory effect on CD44 total and CD44 variant; and this work was published (3).

This has led to a collaboration with Dr. Thomas Flaig (Urologic Oncology) who has access to prostatectomy tissues from patients treated with Silibinin. We used the

luciferase promoter construct to test further the effects of Silibinin. Silibinin did decrease total CD44 and CD44v7-10, suggesting a transcriptional site of action (3).

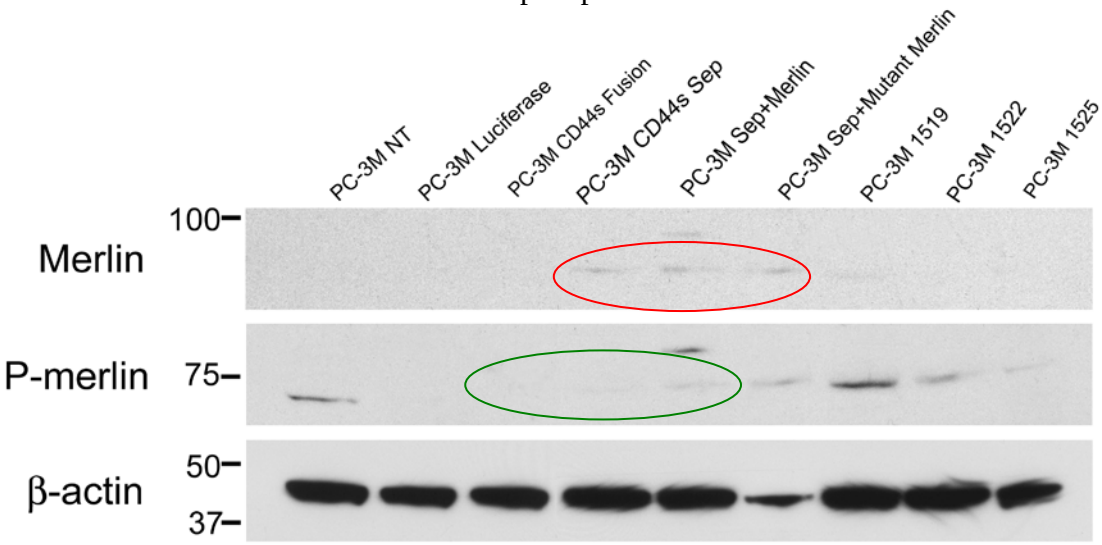
4. Preparation of luciferase constructs for CD44 promoter activation (using a construct from Dr. Jim Lambert of our Department) and for CD44 variant splicing. These have great potential to supplement our work to come. These constructs could be used to verify the extent of CD44 alteration in our cell lines, although we have relied mainly on visualization for the CD44s re-expression transfectants.

In February, the CD44 variant splicing pET construct and empty pET vector were obtained from Dr. Harald Koenig in Germany. They had a CD44v5 splicing construct for use with lymphocytes (23), but we removed it and replaced it with CD44 v7-10 in the multiple cloning site. These constructs were prepared but have not proven effective in our hands and have not been used for almost the past year. The 8 weeks of work done in preparation of the luciferase constructs were detailed in last year's report, Appendix 2.

5. Because of the importance the ezrin-moesin-merlin (ERM) complex in mediating the cytoplasmic signaling of CD44 (24-25), we were interested in how the C-terminal cytoplasmic tail of CD44 interacted with Merlin, a cytoplasmic protein that increases growth in the phosphorylated state and prevents growth in the unphosphorylated state. We postulated that CD44s-Separate protein functions differently from the Fusion protein. The Fusion protein has luciferase bound to its cytoplasmic C-terminus, and so would be unable to interact with Merlin. We are considering growing cells on coverslips and directly visualizing the cytoplasmic versus cell membrane localization of the luciferase. This would provide some indication of whether CD44s-Fusion inserts itself in the cell membrane. Dr. Leila Garcia of U. of Colorado may be able to help us with this.

Plasmid pUHD10-3 that contained *Eco*R1 fragments for either wild-type merlin cDNA or for ineffective, mutant merlin, was a gift of Dr. D. H. Gutmann of Washington University in St. Louis. We grew larger amounts of both, sufficient for transfection. We successfully overexpressed Merlin, and then tested its effect in PC-3M cells with altered CD44 expression (**Fig. 13**). One observation (red oval) was that CD44s Separate cells had increased total Merlin compared to untreated (NT) cells. This was true regardless of whether they were additionally transfected with Merlin or mutant Merlin. A second observation (green oval) was that re-expression of CD44s as a Fusion or Separate protein was associated with decrease in the growth-promoting P-Merlin. The second observation is *confounded* by lack of a signal in the PC-3M luciferase-only cells, so this experiment needs to be repeated. On the other hand, Horiguchi et al. (26) found that silencing of total CD44 dephosphorylates Merlin in benign cells. Therefore, this experiment bears repeating.

Figure 13. Western blot analysis of PC-3M untreated (NT) cells and transfectants for Merlin and phospho-Merlin



6. Because of the interest of some of our colleagues in the Univ. of Colorado Pathology Department's Prostate Cancer Research Laboratory (27) in the tumor suppressive effects of vitamin D (28, 29), we tested the effect of vitamin D and an analog on CD44 expression in $G_s\alpha$-QL and ALVA-3 (a PC-3 derivative) cells. This work was done in 9/07-11/07. Effects of vitamin D or its butyl ester (BE) analog (1α,3ß,24$(OH)_3$-22-ene-24-cyclopropyl-25-n-butyl-ester-vitamin D_3) at 10^{-7} or 10^{-8} M doses, or of the vitamin D receptor antagonist ZK159222 were tested for 24 hr. TaqMan assays for CD44s or CD44v levels gave some hints of a change but results were not consistent. With the use of the above-mentioned *luciferase promoter construct*, we tested whether vitamin D caused CD44 transcription changes in prostate cancer cells. There were no changes. We had thought of including the vitamin D data with the silibinin data (3), but because the result was negative, we limited the paper to Silibinin only.

7. We obtained anti-CD44 v9 producing hybridoma cells from the ATCC and grew these cells to produce mouse monoclonal antibody-containing supernatant. These supernatant stocks replace less-effective 2-3 year-old stocks and will be used for Western immunoblots, and for the immunoprecipitation in Aim 1, second sub-Aim. They will also be used for immunostaining mouse tumor tissue in Aim 2.

8. Another future area of investigation might be to study the role of Early growth response-1 (Egr-1) gene, a transcription factor, in causing altered CD44 transcription and splicing. We have previously published on Egr-1 (30) and recent work has implicated Egr-1 in regulation of CD44 (31).

REPORTABLE OUTCOMES:
Manuscripts: Three manuscripts have been published (1-3). The PDFs are in the Appendix. A fourth is in preparation and is in the Appendix.

Presentations: A presentation of these data was made by Dr. Iczkowski today, April 29, 2009, to the Hormone-Related Malignancies conference of the Oncology Department.
.
Cell lines: We have established stable cell lines with CD44 variant RNAi, and as controls, CD44 variant RNAi with 4 conservative mutations. We have cells overexpressing CD44s as a separate protein or as a fusion protein with luciferase. All will be used for Aim 2.
Grant proposals submitted: A DOD pre-proposal was submitted in March 2009, to pursue the microRNA project, on the strength of our published findings (2). However, we were not invited to submit a full proposal.

CONCLUSION: The past year we had publications resulting from Aim 1 (1), and from tangentially related projects on the influences of microRNA (2) and Silibinin (3) on CD44 in prostate cancer. We now have performed or are performing several *in vitro* invasion and proliferation assays that our laboratory had not done previously. These assays expand the original sub-aims of Aim 2 by documenting a number of in vitro and in vivo effects of altering CD44 expression. Both in vitro and in vivo experiments thus far suggest that re-expression of CD44s holds promise for gene therapy, alter morphology and cell adhesion, and can reduce prostate cancer growth and invasion.

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APPENDICES:

Appendix 1. Metabolic Labeling Study. By .Dr. Alina Handorean
Appendix 2. Our three paper publications
Appendix 3. Our current manuscript draft.

Research report
Metabolic labeling of proteins

The metabolic labeling of proteins of PC3M cells nontreated and PC3M cells overexpressing CD44s was used as a tool to discriminate the expression level of proteins in the two cell types. CD44s overexpressing cells should have a lower level of expression of CD44v9 proteins, fact that can be illustrated by using phosphor imaging that will provide information on the decrease in the band corresponding to this protein in PC3M cells overexpressing CD44s.

From the information available form the PDB (Protein Data Bank) we found out that the hyaluronan binding domain of human CD44(1poz) that consists of 150 amino acids has 3 methionine residues and 6 cysteine residues that can be potentially labeled by using the [35S] Met [35S]Cys mixture from Perkin Elmer. The whole protein contains 742 amino acids, and consists of 14 residues of mehionine and 9 residues of cysteine. (Protein ID P16070).

The cells overexpressing CD44s and the PC3M control cells were grown in 6-well plates in RPMI medium + 10% dialyzed fetal bovine serum (FBS) until they were 80-90% confluent. Prior to the radiolabeling step, the medium was replaced with methionine-deficient RPMI 1640 medium +10 % dialyzed FBS and the cells were allowed to grow for 30 minutes at 37°C in a 5% CO_2 incubator. 25µCi of the radioactive mixture were added to 1 mL of RPMI medium and allow the cells to incorporate radioactive methionine and cysteine. After 1 hour, the radioactive medium was replaced by RPMI depleted medium that contained 100µg/mL cold cysteine and 100µg/mL cold methionine. The proteins were extracted using RIPA buffer and the amount of radioactivity incorporated was checked by running an SDS-PAGE gel followed by exposure to the cassette and phosphor imaging. No band could be detected, so we concluded that the amount of time the cells were in contact with the radioactive mixture was insufficient for the incorporation of radioactive material during protein synthesis.

We performed a time course reaction permitting the cells to be in contact with the radioactive mixture for a longer time and we determined by phosphor imaging that the one hour incubation used previously was insufficient and the optimum time for labeling is 16 or 24 hours. (Figure 1)

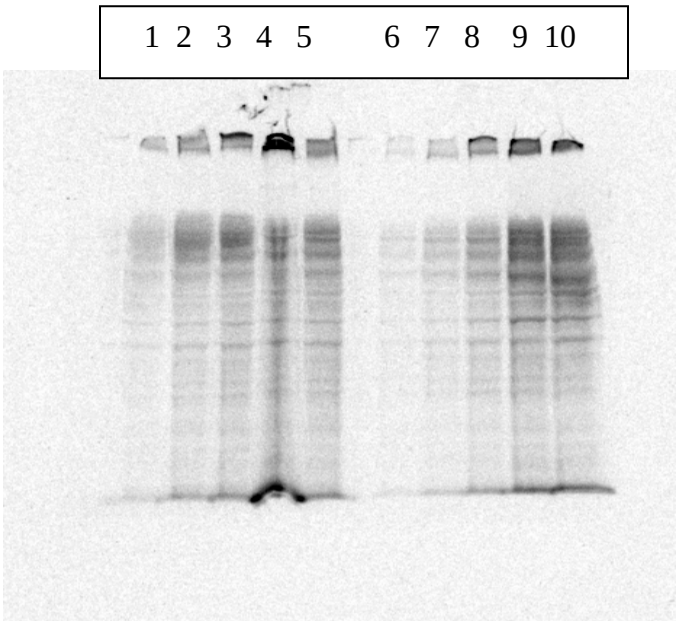

Figure 1 legend:
Lane 1-5: PC3M untreated cell lysate
Lane 6-10: PC3M overexpressing CD44s (luciferase construct)
Lanes 1 and 6: 1 hr exposure
Lanes 2 and 7: 2 hrs exposure
Lanes 3 and 8: 6 hrs exposure
Lanes 4 and 9: 16 hrs exposure
Lanes 5 and 10: 24 hrs exposure

We also optimized the amount of radioactivity added to the cell culture. Initially, we added 25 µCi of radioactively labeled mixture to 1 mL of culture medium. We treated cells with 10, 25, 35 and 50 µCi/mL but no significant difference in terms of the radioactive labeling of proteins was observed for these samples compared to the 25µCi/ml we initially used.

For the next step, we decided to use the 24 hours cell lysate extracts for the immunoprecipitation(IP) assay. 50-100 µg of cell lysate were incubated overnight at 4°C with 100 µL of the CD44v9 culture supernatant previously grown according to the manufacturer's procedure. The protein-antibody complex was further incubated with 70 µL of protein G agarose beads, overnight at 4°C with end-over-end mixing. The solution is centrifuged at maximum speed for 1 minute and the supernatant is discarded. The precipitate is washed two times in washing solution and eluted with 4X NuPAGE buffer. Followed the centrifugation, the beads will precipitate and the protein-antibody complex remains in the supernatant and it is ready to be loaded on SDS-PAGE. Followed the exposure to the cassette and the phosphor imaging of the screen, no band could be detected for the expected 70 kDa molecular weight. (Figure 2)

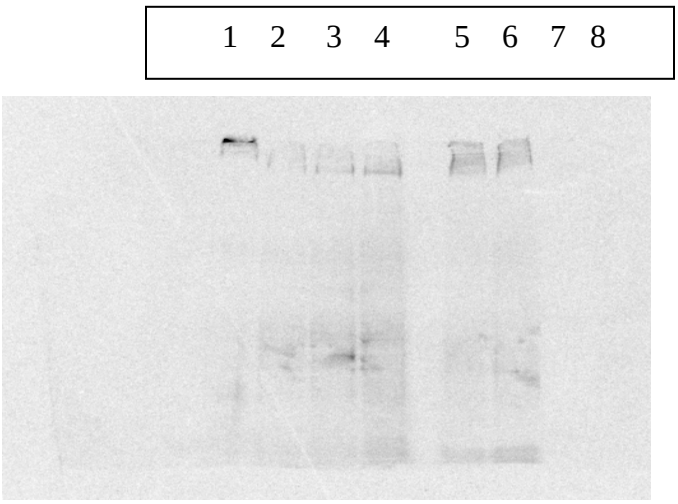

Figure 2 legend:
Lane 1-4: PC3M non treated cell lysate
Lane 1: 10µCi radioactive mix
Lane 1: 25µCi radioactive mix
Lane 1: 35µCi radioactive mix
Lane 1: 50µCi radioactive mix

Lane 5: PC3M non treated cell lysate 8 hrs exposure to radioactive mixture
Lane 6: PC3M overexpressing CD44s cell lysate 8 hrs exposure to radioactive mixture

Lane 7: PC3M non treated IP sample 8 hrs exposure to radioactive mixture
Lane 8: PC3M overexpressing CD44s IP sample 8 hrs exposure to radioactive mixture

We definitely proved that the proteins of both type of cells were radioactive labeled and we know from dot-blot experiments and other western blots run in the lab that the antibody against CD44v9 is working properly and is able to bind the protein of interest. Our conclusion is that the amount of radioactivity for this particular protein we expect to show as a 70 kDa band is not enough to discriminate in between the treated and untreated cells. To confirm this conclusion, we performed measurements of the amount of radioactivity incorporate in cell lysate by liquid scintillation counter. For all samples, treated and untreated, the calculated amount of radioactivity incorporated ranged from 2-10%, that corresponds to the amount reported in the literature.(1). However, this low percentage prevented us to distinguished the treated from untreated samples relative to the expression levels of CD44v9.

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MAP kinase pathways and calcitonin influence CD44 alternate isoform expression in prostate cancer cells

Eric W. Robbins [1], Emily A. Travanty [1], Kui Yang [1] and Kenneth A. Iczkowski [1]

[1]From the Department of Pathology, Anschutz Medical Campus, University of Colorado Denver, Aurora, Colorado, USA.

Corresponding author:
Kenneth A. Iczkowski, M.D., Pathology—P.O. Box 6511, UCHSC—Campus Mail Stop 8104, Aurora, CO 80010. Tel: 303-724-0155, Fax: 303-724-3712, E-mail: Kenneth.Iczkowski@UCDenver.edu

Abstract

Background: Dysregulated expression and splicing of cell adhesion marker CD44 is found in many types of cancer. In prostate cancer (PC) specifically, the standard isoform (CD44s) has been found to be downregulated compared with benign tissue whereas predominant variant isoform CD44v7-10 is upregulated. Mitogen-activated protein kinase pathways and paracrine calcitonin are two common factors linked to dysregulated expression and splicing of CD44 in cancer. Calcitonin has been found to increase proliferation and invasion in PC acting through the protein kinase A pathway.

Methods: In androgen-independent PC with known high CD44v7-10 expression, CD44 total and CD44v7-10 RNA or protein were assessed in response to exogenous and endogenous calcitonin and to inhibitors of protein kinase A, MEK, JNK, or p38 kinase. Benign cells and calcitonin receptor-negative PC cells were also tested.

Results: MEK or p38 but not JNK reduced CD44 total RNA by 40%-65% in cancer and benign cells. Inhibition of protein kinase A reduced CD44 total and v7-10 protein expression. In calcitonin receptor-positive cells only, calcitonin increased CD44 variant RNA and protein by 3 h and persisting to 48 h, apparently dependent on an uninhibited p38 pathway. Cells with constitutive CT expression showed an increase in CD44v7-10 mRNA but a decrease in CD44 total RNA.

Conclusions: The MEK pathway increases CD44 RNA, while calcitonin, acting through the protein kinase A and p38 pathway, facilitates variant splicing. These findings could be used in the formulation of therapeutic methods for PC targeting CD44 alternate splicing.

Background

CD44, a transmembrane glycoprotein, is the product of a gene that can undergo extensive alternate splicing. The standard (**CD44s**) isoform is ubiquitous but tissue-specific isoforms may include an assortment of 10 variant (**v**) exons (**CD44v**). CD44 facilitates multiple cellular functions. CD44 enables cell-cell and cell-matrix adhesion--primarily to its main ligand hyaluronan, and links the cell membrane to the actin cytoskeleton, modulating motility. CD44 is universally dysregulated in human cancer, and this imbalance of isoforms allows tumor growth and invasion[1-8]. CD44v are expressed in prostatic secrectory cells while CD44s is found in the whole epithelium. About 30% of cases of prostate cancer (PC) undergo a transition from quiescent to aggressive. Altered CD44 and other adhesion molecules permit this transition in which tumor cells detach, interact with proteins that digest stromal matrix, migrate through matrix, and intravasate into lymphovascular channels.

By isolating RNA from clinical PC specimens, we discovered that the major variant isoform expressed in PC is CD44v7-10. This PC signature was consistently present in both primary and metastatic PC[1-3]. Interference against this CD44v caused a 69% reduction in invasion index compared to untreated control cells[3]. Moreover, PC loses the splicing ability to produce the CD44s expressed in benign prostate[3,9,10]. CD44 must oligomerize to bind matrix ligands or to cause metastasis[11] and variant isoforms, with longer extracellular tails, have altered ability to complex[12]. We found that the CD44v7-10 isoform makes PC cells preferentially bind to fibronectin rather than hyaluronan; re-expression of CD44s causes the predominant ligand to revert from fibronectin back to hyaluronan[4]. In mouse xenografts of PC-3 prostate tumor, forced expression of CD44s reduced growth *in vitro* and tumorigenicity[5], and our use of RNAi against CD44v7-10 in xenografts yielded similar effects (unpublished results).

In PC, calcitonin (CT) acts as a paracrine growth factor that up-regulates CD44 variant[4, 6]. In histologic specimens PC, but not benign secretory epithelium, contains CT[13] and its receptor (CTR)[14], and CT exerts paracrine effects that promote proliferation[15], invasion[16], and metastasis[17]. CTR, essential for prostate cancer tumorigenicity[18], is coupled to the transduction protein $G_s\alpha$. We have shown that CT promotes alternate splicing leading to CD44v7-10 mRNA and protein[4,6] by acting through $G_s\alpha$ signaling[3]. $G_s\alpha$ stimulates the cyclic AMP signaling cascade[17,19] and protein kinase A (PKA)[16].

PKA, in turn, acts on the 3 main MAPK pathways: a growth factor-responsive pathway that uses MAP2K (also called MEK) as key downstream effector; and two stress-activated pathways, c-jun N-terminal kinase (JNK), and p38 kinase, that respond to stress including cytokines, osmotic shock, and irradiation. CD44 variants activate MAPK pathways[20], sometimes by functioning as co-receptors for growth factors[21]. MAPK pathways, in turn, can cause CD44 alternative splicing to include variant exons[22]. Oncogenes such as ras[7,23] and mitogens using the MEK-ERK MAP kinase (MAPK) pathway[7], but not the p38 pathway[24], induce CD44 promoter activity and increase expression of certain CD44v. To test whether these influences modulate RNA levels and alternative splicing of CD44 in PC, we studied the CT signaling system, PKA, and MAPK pathways. CD44 mRNA and protein levels were measured.

Methods

Cell lines

PC-3 cells (American Type Culture Collection, Manassas, VA) were incubated in F12-K medium, 10% fetal calf serum, and antibiotics at 37ºC in a 5% CO_2 incubator. $G_s\alpha$-QL cells, CT+, CT-, and CTR- cells were gifts of Dr. Girish Shah, Univ. of Louisiana-Monroe[17]. The $G_s\alpha$-QL cells were derived from metastasizing PC-3M cells stably transfected by a plasmid that directs expression of mutant, constitutively active $G_s\alpha$[17,19]. These three cell lines were grown

in RPMI 1640 with L-glutamine, 5% fetal calf serum, 15% horse serum and antibiotics. Benign BPH-1 cells (from Dr. Simon Hayward, Vanderbilt Univ., Nashville, TN) were grown in RPMI with 10% fetal calf serum and antibiotics. For each experiment, cells in a flask were trypsinized and saline washed to remove trypsin. 200,000 cells were plated per well on a 6-well plate. Cells were adherent and 80% confluent for all experiments. Each treatment was applied to three wells, with three other wells as mock treated controls.

Effect of exogenous calcitonin

PC-3M cells express both CT and CT-receptor (CTR)[16]. To test the effect of solely exogenous CT on CD44, we used the derivative, CT-minus (CT-). CT- cells have endogenous CT stably knocked down to undetectable levels using anti-CT hammerhead ribozymes[25]. Salmon CT (BAChem, Torrance, CA) was used at a physiologic 50 nM dose[14,16], which effectively alters CD44[6], or at 250 nM. The K_d of CTR is 4-21 nM[15]. To detect acute versus long-acting effects on RNA and protein levels, cells were treated with 50 nM CT and harvested after 3 h or 48 h. To determine whether CT effects on CD44 proteins resulted from *de novo* protein synthesis versus protein stabilization, cycloheximide (10 nM) was given to CT- cells after 3 hr of CT exposure, and cells harvested 1 hr, 3 hr, 6 hr, and 9 hr subsequently, similar to a prior CD44 study[26]. Also tested were highly invasive, CT positive $G_s\alpha$-QL cells[17]. Finally, to rule out non-CTR-mediated CT effects, two negative controls were tested: PC-3 cells (shown to be negative for CT receptor[14]), and cells called CTR-, derived from PC-3M cells after anti-CT receptor ribozyme knockdown of CTR[18]. CTR- cells have very low levels of CD44v protein[4].

Effect of endogenous calcitonin

To test the effect of endogenous CT on CD44 RNA, we used PC-3M cells expressing CT-pcDNA 3.1 plasmid (CT+), which constitutively express CT[25]. 125,000 PC-3M or CT+ cells per well were plated and allowed to grow for 72 hours.

Inhibition of protein kinase A and MAPK components

$G_s\alpha$-QL cells were chosen for these studies because they have the highest baseline CD44v[3]. Protein kinase A inhibitor H-89 (in 50% ethanol) (Calbiochem, La Jolla, CA) was added to cells in fresh medium (1 mL/well) at 1 µM, previously shown effective in nerve cells[23] or at a 10 µM dose. Cells were incubated with H-89 for 24 hours[27] then harvested.

In similar assays, either 10 µl of 1 mM JNK inhibitor (SP600125, Calbiochem) or 12.5 µl of 2 mM MEK inhibitor (PD98059, Calbiochem) in water were added, yielding concentrations previously shown effective: 10 µM for JNK[28] and 25 µM for MEK (personal communication, Dr. Bolin Liu). p38 kinase inhibitor (SB203580, Calbiochem) was used at 10 µM[29,30] in DMSO, and control cells received DMSO only. Cells were incubated for the optimum time of 48 h[28] to show effects.

Interaction of CT with MAPK pathways

Based on results above, we tested the effect of pretreatment with MEK or p38 kinase inhibitors on CT-mediated alteration of CD44 expression. 25 µM MEK inhibitor or 10 µM p38 kinase inhibitor was added to CT- cells 4 hours prior to administering 50 nM CT. Cells were harvested after 48 h as above.

Real time TaqMan RNA analysis

Total RNA was prepared from cell pellets using Trizol (Invitrogen, Carlsbad, CA) as described by the manufacturer. RNA was further purified by isopropanol precipitation, resuspended in RNAse-free water, and its concentration measured. Complementary DNA (cDNA) was synthesized from 4 µg total RNA in 20 µl reaction mixture as we did previously[9]. At least triplicate samples were run using a primer/probe set for <u>all</u> CD44v that brackets the entire variant region[6], one for CD44 total that binds a standard exon, and 18S ribosomal RNA. Quantitative PCR reactions were optimized to 4 µg cDNA (0.16 µg with 18S) plus the manufacturer's master mix and primer/probe sets (Applied Biosystems, Foster City, CA) in a volume of 20 µl. The amplification protocol was as follows: hold 50ºC 2 min, 95ºC 10 min, then

40 cycles of (95ºC for 0:15 and 60ºC for 1:00) using the ABI Prism 7700 cycler (Perkin-Elmer, Waltham, MA). Primer/probe sets for CD44v were: forward, AACGCTTCAGCCTACTGCAAA; reverse, TCTTCCAAGCCTTCATGTGATG; probe, GATTTGGACAGGACAGGACCTCTTTCAATG. For CD44 total we used forward, CAACTCCATCTGTGCAGCAAA; reverse, GTAACCTCCTGAAGTGCTGCTC; probe, CATATTGCTTCAATGCTTCAGCTCCACCTG. Primer and probe sets for 18S were proprietary to the manufacturer.

Western blot analysis

Cultured cells were directly lysed in their wells using RIPA buffer (Upstate Biologicals, Lake Placid, NY) with protease inhibitor Complete-mini tablet (Applied Science, Indianapolis, IN). Protein concentration of the cell lysate was estimated by Bradford method. Samples were resolved on SDS-PAGE using 25 µg sample/lane with the NuPAGE system (Invitrogen, Carlsbad, CA). 5 µl of Rainbow protein marker (RPN 756, Amersham, Piscataway, NJ) was run in at least one lane. After electrophoresis for 2 hr, the protein was transferred to PVDF. Three primary antibodies were used. To assess CD44v9 (the largest component of the overexpressed CD44v7-10) the membrane was reacted with neat supernatant from the hybridoma cell line HB-258 (ATCC). CD44 standard was assessed using anti-HCAM (DF1485, Santa Cruz Biologicals, Santa Cruz, CA, 1:2000), which binds all CD44 isoforms. Anti-β-actin antibody (Sigma, St. Louis) was used at a dilution of 1:10,000. Membranes were washed 3 x 15 min in TBS with 20mM Tris pH 7.5 and 1:1000 dilution of goat anti-mouse IgG antibody labeled with biotin (Bio-Rad) was added at 1:9000 dilution in 5% skim milk for 1 hr. Reactivity was detected using a chemiluminescent system (SuperSignal West Pico Substrate, Pierce Biotechnology, Rockford, IL). Each experimental run was conducted at least twice.

Statistical analysis

TaqMan data were analyzed by the $2(-\Delta\Delta C_T)$ method [31] to determine fold change in gene expression (mock treated cells=1.00). The ΔC_T was taken as the difference between the CD44v or CD44 total and the 18S ribosomal RNA C_Ts. The $\Delta\Delta C_T$ was obtained using the mean ΔC_T of mock treated cells as calibrator. Each TaqMan result was compared to 1.00 using 2-tailed paired t-test. Statistical significance was set at $p < 0.05$.

Results

Calcitonin increases CD44v

In the PC-3M-derived CT- cells, a 50 or 250 μM CT dose after 48 h had little effect on the total amount of CD44 RNA, but the CD44v was tripled (**Fig. 1a**). Although different binding affinities of primer/probe sets preclude determining CD44v as a percent of CD44 total, the *relative* percent of CD44v RNA can be calculated by the $2(-\Delta\Delta C_T)$ method, as increasing fivefold after 50 μM CT. The same response, but less marked, was seen in $G_s\alpha$-QL cells, at 50 and 250 μM doses. In CTR- cells and PC-3 cells—both lacking CTR—exogenous CT had little effect. Similarly, BPH-1 cells responded to CT with very slight stimulatory effect on CD44v, and no effect on CD44 total. At the protein level, however, the CT- cells treated with CT showed increases in both total and variant CD44 after just 3 h (**Fig. 1b**) and at 48 h (**Fig. 1c**). The stimulation of CD44v protein was attenuated by cycloheximide up to 9 h after CT (data not shown). This suggests that *de novo* protein synthesis is required and that upregulation of CD44v is not simply a result of protein stabilization.

In CT+ cells, CD44v mRNA doubled compared to PC-3M while CD44 total expression was cut in half (**Fig. 1d**). This suggests that endogenous CT exerts an increase on CD44 variant similar to exogenous CT.

Protein kinase A and MAP kinase pathways and their interaction with calcitonin

$G_s\alpha$-QL cells have high basal levels of CD44v7-10; for this reason, these cells were used to examine the effects of protein kinase A (PKA) and MAPK pathway inhibitors. PKA inhibitor lowered CD44 total and CD44v mRNA (**Fig. 2a**) and dose-dependently decreased protein for both (**Fig. 2b**). Downstream to PKA, inhibition of MEK significantly decreased CD44 total ($p=0.001$) and non-significantly decreased CD44v. In contrast, inhibition of JNK had no significant effects. p38 inhibitor led to a larger, significant decrease in CD44 variant and a smaller significant decrease in CD44 total (**Fig. 2a**). MEK and JNK inhibitors were also tested in PC-3 cells and had no effect (data not shown). MEK inhibitor was also tested in BPH-1 cells, in which it reduced CD44 total and variant RNA.

To examine the dependence of CT effects on MAPK pathways, the CT- cells were pretreated with p38 inhibitor 4 h prior to administration of CT. Results were similar to p38 inhibitor alone: more than 50% decrease in CD44 total but none in CD44v (**Fig. 3**). This lack of CD44v suppression contrasts with p38 inhibitors marked CD44v suppression in $G_s\alpha$-QL cells (**Fig. 2a**), which have far higher CD44v[4]. This suggests that CT mediated splicing is through p38 kinase. In further support of this, the expected CT induced tripling of CD44v mRNA in CT- cells (**Fig. 1a**) was prevented by p38 inhibitor pretreatment. Pretreatment with MEK inhibitor before CT also blunted the expected rise in CD44 variant mRNA seen in **Fig. 1a**, and JNK inhibitor pretreatment had no effect (data not shown).

Discussion and conclusion

Here, we demonstrate that calcitonin (CT) causes CT receptor-dependent increases in CD44 alternate splicing in prostate cancer (PC), apparently mediated through p38 kinase. Furthermore, transcription but not splicing appears to require the MEK/ERK (MAPK) pathway. Proposed interactions are shown (**Fig. 4**).

Paracrine CT is among several growth factors that interact with CD44[22]. In our prior *in vitro* studies up to 100 nM exogenous CT[6], or CT originating endogenously (in a PC-3 derivative called CT+ [4]), increased CD44v7-10 expression at the mRNA and protein levels. This was also observed in LnCaP, PC-3, and PC-3M derived cells; however, we had not examined total CD44 previously. Here, we used CT-minus (CT-) cells, an androgen-independent PC-3M derivative, to exclude all endogenous CT influence, so any effects would be attributable solely to exogenous CT. In CT- cells, the aberrant splice product is CD44v7-10[6]. This action occurred also in $G_s\alpha$-QL cells, which are CTR+. Finally, CT+ cells showed an increase in CD44v compared with PC-3M cells. Supporting the view that this stimulation was CT-receptor mediated and not nonspecific, administering CT to CT-receptor negative PC-3 or CTR- cells did not have this effect. In further support of this interpretation, with benign BPH-1 cells, which are also negative for CT and CTR (personal communication, GV Shah), exogenous CT exerted no effect.

It is a novel observation that CT increases CD44v mRNA and protein as early as 3 h in cells that are CTR+, and has little effect on CD44 total. A response to CT should occur in the first several hours (personal communication, GV Shah), and indeed these increases were evident in CD44 protein levels at 3h and 48 h.

Since the MEK/ERK pathway and the two stress-activated MAPK pathways are implicated in androgen-independent prostate cancer growth[32], we tested all three for modulation of CD44. We had found that CD44v7-10 protein was overexpressed in xenografts of $G_s\alpha$-QL compared to PC-3M[3]; and i*n vitro*, pharmacologic stimulation of $G_s\alpha$ or adenylyl cyclase raised CD44v7-10[6]. Using a PKA inhibitor, we found reduced total and CD44v7-10 mRNA, suggesting involvement of MEK pathway. Over half of PC cases have activated MEK-ERK signaling, shown by immunohistochemistry for p44/ERK1 and p42/ERK2[33].

To assess MAPK inhibitor effects, we chose $G_s\alpha$-QL cells, derived from metastasizing PC-3M cells that stably express *gsp* mutant, constitutively active $G_s\alpha$[17,19] because they have high baseline CD44v7-10[3,6]. We found MEK inhibitor caused similar percent decreases in CD44 total and CD44 variant, implicating MEK in transcription if not CD44v splicing. Similar effects have been noted with ras oncoprotein, which acts on the MEK-ERK pathway[24]. Ras activation can induce CD44 promoter activity in fibroblasts, as shown using transient cotransfection of c-ras expression constructs and CD44 promoter reporter gene constructs[7]. Leakiness of splice control is proposed to lead to increased CD44v[7]. In activated T-lymphocytes during the immune response, mutant *ras* stimulation of MEK-ERK pathway increases CD44 total mRNA and triggers inclusion of CD44v exons in the mature RNA[24]; from our experiments, MEK seems to be active at least in CD44 transcription in $G_s\alpha$-QL cells.

Some studies have suggested a positive feedback loop coupling MEK/ERK pathway and CD44v splicing. Activation of *ras* oncogene in rat fibroblasts[7] and HeLa cervical cancer[23] and of its effector, the MAP kinase pathway in T-cells[24] both upregulate CD44v splicing. CD44 variants, in turn, serve as coreceptors for growth factor receptors that activate ras[23] or form complexes with receptor tyrosine kinases such as c-met[8,21] to mediate cell signaling. Moreover, CD44v6 promotes T-cell proliferation by persistently activating MAP kinases[20], and CD44v8-10 causes apoptosis resistance in small cell lung cancer by activating Rho-stimulated focal adhesion kinase (FAK)[34].

We examined p38 kinase in benign and PC cells. In BPH-1, CD44 total RNA decreased with p38 kinase inhibition but variant form was unchanged. Since benign prostate lacks the aberrant splicing leading to CD44v7-10[3], but CD44v3-10 expression is present[35], the latter may be the form detected in benign cells. In $G_s\alpha$-QL cells, a more marked effect on total and variant CD44 was seen. However, p38 may have CT-independent actions. Similar to our current

and previous[6] findings with CD44 and $G_s\alpha$, p38 and MEK (but not JNK) were responsive to G-protein-coupled P2Y purinoceptor agonist ATP in PC-3 cells (CTR-negative), and these 2 pathways were required for invasion[30]. p38 has recently been recognized as a cell proliferation and survival factor in PC[36] partly by regulating IL-6 secretion[32]. Taken together with our findings about the MEK/ERK role in CD44 transcription, this could reflect convergence of the ERK1/2 and p38 systems in activating the MNK1 kinase, which enhances transcription of certain targets[37], suggesting a common final pathway that stimulates CD44 expression in PC.

We tested possible JNK pathway effects on CD44, not previously examined in the literature. JNK appears mainly important in PC apoptosis[29,38] and promoting chemotherapy susceptibility. JNK inhibitor slightly decreased CD44 total protein and did not change CD44v mRNA or protein in $G_s\alpha$-QL cells. Our data suggest that JNK is a minor influence on CD44 expression.

Inhibition of p38 and MEK pathways affected CD44 in $G_s\alpha$-QL cells. To investigate whether either might mediate CT's effects on CD44, we administered CT to CT- cells after blocking either one of these pathways. CT- cells have low baseline CD44v[6], and p38 inhibitor did not suppress the CD44v, but it blocked the expected stimulation of CD44v by CT, suggesting that p38 mediates CT-stimulated alternative splicing. The marked CD44v suppression seen in $G_s\alpha$-QL cells, which have endogenous CT and high baseline CD44v, adds support for this interpretation. It is tempting to speculate that CT signaling, raising cAMP, may act through the effector, "exchange factor directly activated by cAMP" (Epac, **Fig. 4**). Epac has been shown to activate p38 kinase and mobilize intracellular calcium in neurons[39]. This PKA-independent mechanism would explain why PKA affected primarily CD44 transcription, yet p38 showed evidence of an additional effect on splicing.

To our knowledge, this is the first report in PC of how interactions between CT, and MAP kinase pathways, dysregulate the expression and splicing of the CD44 molecule. CD44 variant isoforms, probably through alterations in multimerization[12] and ligand binding[4], allow prostate cancer invasion[3,6]. This knowledge may find application in targeting the aberrant splicing of CD44 in PC by gene therapy, molecular inhibitor therapy, or for sensitization to radiotherapy.

Abbreviations: CD44: cell determinant 44; CT: calcitonin; CTR: calcitonin receptor; ERK: extracellular signal-regulated kinase; JNK: Jun N-terminal kinase; MAPK: mitogen-activated protein kinase; PC: prostate cancer; PKA: protein kinase A

Competing interests: Financial: none; Non-financial: none.

Authors' contributions: EWR and EAT conducted experiments; EAT performed statistical analysis; KY did some western blot analyses; KAI conceived of the study and wrote much of the manuscript.

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Figure legends

Fig. 1a. RNA response to exogenous CT at 48 h is calcitonin (CT) receptor-dependent. CD44 total and variant RNA are quantified by triplicate TaqMan RT-PCR experiments. In benign BPH-1 cells, no response is observed to exogenous calcitonin (CT). CT- cells, a PC-3M derivative that lack endogenous CT but have CT receptor, showed a decrease in CD44 total RNA but an increase in variant RNA. The $G_s\alpha$-QL cell derivative showed the same trend. In CT-receptor-negative CTR- and PC-3 cells, there is no significant response to CT. Error bars are standard deviation. *p=0.01; † p=0.05 with respect to mock treated controls.

Fig. 1b. Western blot analysis. Exogenous calcitonin given to the CT- cells for 3 h stimulates expression of CD44s at 75 kD (top), and CD44v at 180 kD and its cleavage products below 97 kD. Comparison is shown to cells with no treatment (NT). β-actin analysis control confirms equal protein loading (bottom).

Fig. 1c. This stimulatory effect persists at 48 h and at doses of 50 mM or 250 mM exogenous calcitonin. Also, a time-dependent reduction in protein was observed after cycloheximide treatment, suggesting that CT effect on CD44 variant requires *de novo* protein synthesis and does not result from protein stabilization.

Fig. 1d. CT+ cells, endogenously expressing CT, are derived from PC-3M cells and have increased RNA for CD44v (p=0.02) but a decrease in CD44 total (p=0.008).

Fig. 2a. RNA response to molecular inhibitors. CD44 total and variant RNA in triplicate TaqMan RT-PCR experiments. In subconfluent $G_s\alpha$-QL prostate cancer cells, significant decreases of about 50% or more were observed after protein kinase A (PKA) inhibitor H-89, or

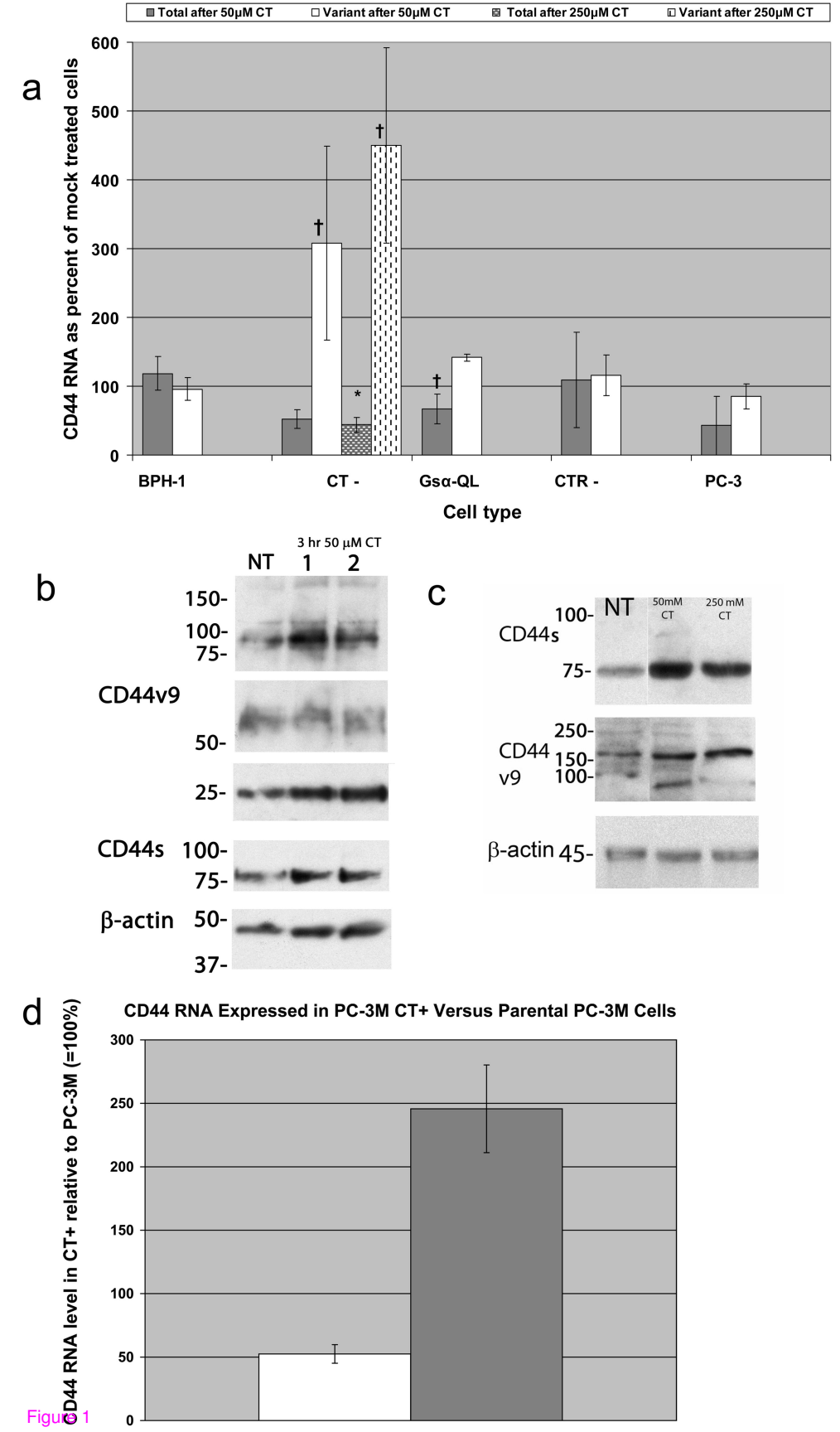
inhibitors of downstream signaling pathways MEK or p38 kinase, but not JNK inhibitor. Error bars are standard deviation. *p=0.01; **p=0.001; †p=0.0002; ***p=0.0001 with respect to mock treated controls.

Fig. 2b. Western blot analysis. Protein kinase A inhibitor exerts a dose-dependent decrease on CD44s and CD44v compared with EtOH control in $G_s\alpha$-QL cells.

Fig. 3. In CT- cells, the blockage of p38 kinase counteracts exogenous calcitonin (CT) stimulatory effect on CD44 variant RNA expression. Triplicate TaqMan RT-PCR experiments. While p38 blockade does not affect the CT-induced decrease in CD44 total, it does abrogate the expected tripling (Fig. 1) in CD44 variant. Conversely, MEK inhibitor moderately reduced total and variant CD44, and this effect was not counteracted by CT, suggesting CT does not act downstream of MEK. *p=0.02; **p=0.006 with respect to mock treated controls.

Fig. 4. Proposed effects of calcitonin and MAP kinase pathways on CD44 expression in androgen-independent prostate cancer.

Calcitonin (CT) binds to its receptor (CTR), which is coupled to the $G_s\alpha$ transduction protein. $G_s\alpha$ activity, mediated through cAMP, activates protein kinase A (PKA) [19,25]. PKA activates the MAPK kinase (MEK)-extracellular regulated kinase (ERK) pathway, that facilitates CD44 transcription. CT also induces splicing of CD44 to include v7-10, dependent on p38 but not on PKA. p38 may be induced by Exchange protein activated by cAMP (Epac). p38 could affect splicing machinery directly, through other downstream effectors, or by causing release of intracellular Ca^{2+}.


Total after 50μM CT
Variant after 50μM CT
Total after 250μM CT
Variant after 250μM CT
a
CD44 RNA as percent of mock treated cells
600
500
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BPH-1
CT -
Gsα-QL
CTR -
PC-3
Cell type
b
3 hr 50 μM CT
NT
1
2
150-
100-
75-
CD44v9
50-
25-
CD44s
100-
75-
β-actin
50-
37-
c
NT
50mM CT
250 mM CT
100-
CD44s
75-
250-
CD44 v9
150-
100-
β-actin
45-
d
CD44 RNA Expressed in PC-3M CT+ Versus Parental PC-3M Cells
CD44 RNA level in CT+ relative to PC-3M (=100%)
300
250
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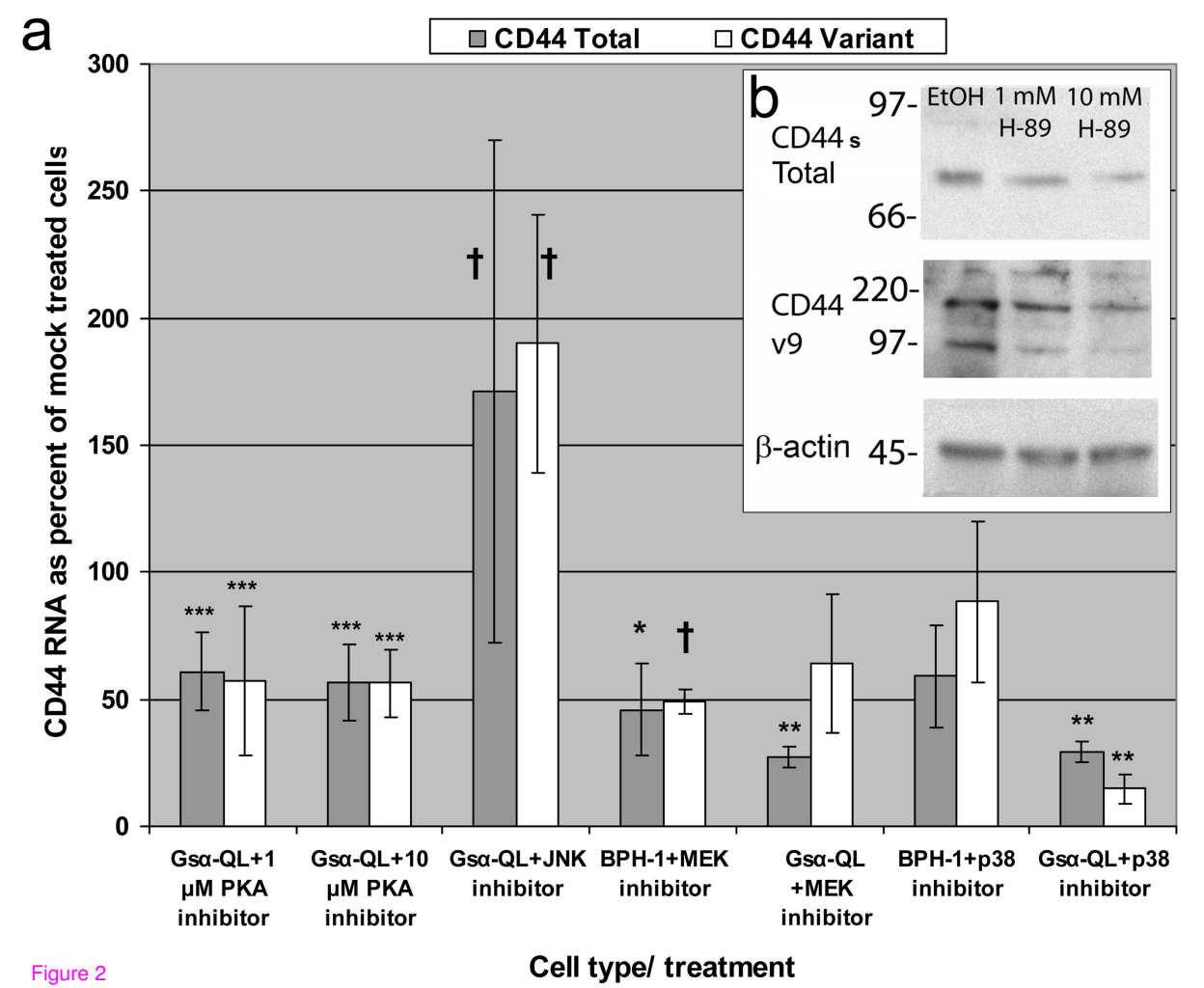
Figure 1


a
CD44 Total
CD44 Variant
CD44 RNA as percent of mock treated cells
300
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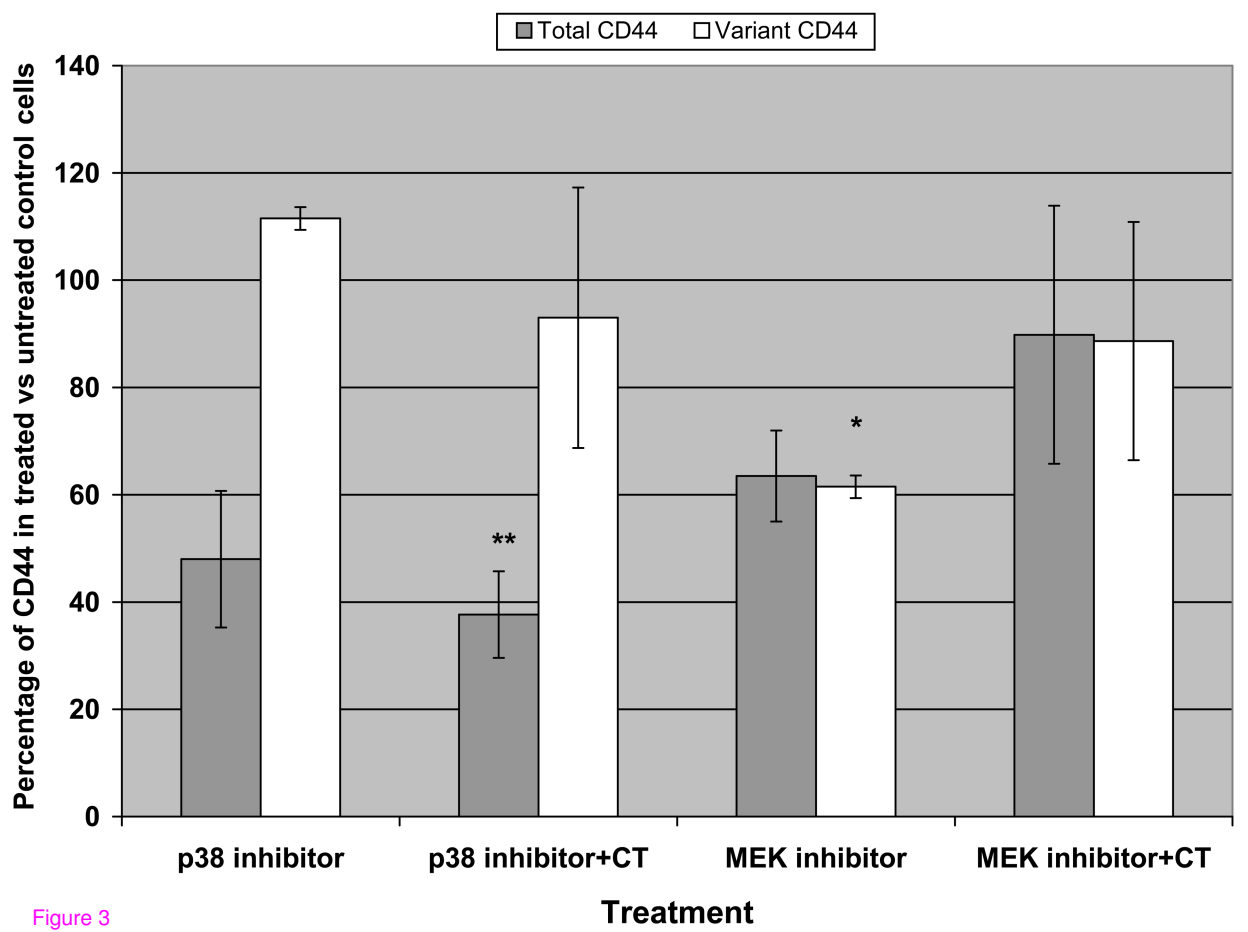
Gsα-QL+1 μM PKA inhibitor
Gsα-QL+10 μM PKA inhibitor
Gsα-QL+JNK inhibitor
BPH-1+MEK inhibitor
Gsα-QL +MEK inhibitor
BPH-1+p38 inhibitor
Gsα-QL+p38 inhibitor
Cell type/ treatment
b
EtOH
1 mM H-89
10 mM H-89
CD44 s Total
97-
66-
CD44 v9
220-
97-
β-actin
45-

Figure 2


Total CD44
Variant CD44
Percentage of CD44 in treated vs untreated control cells
140
120
100
80
60
40
20
0
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*
p38 inhibitor
p38 inhibitor+CT
MEK inhibitor
MEK inhibitor+CT
Treatment

Figure 3

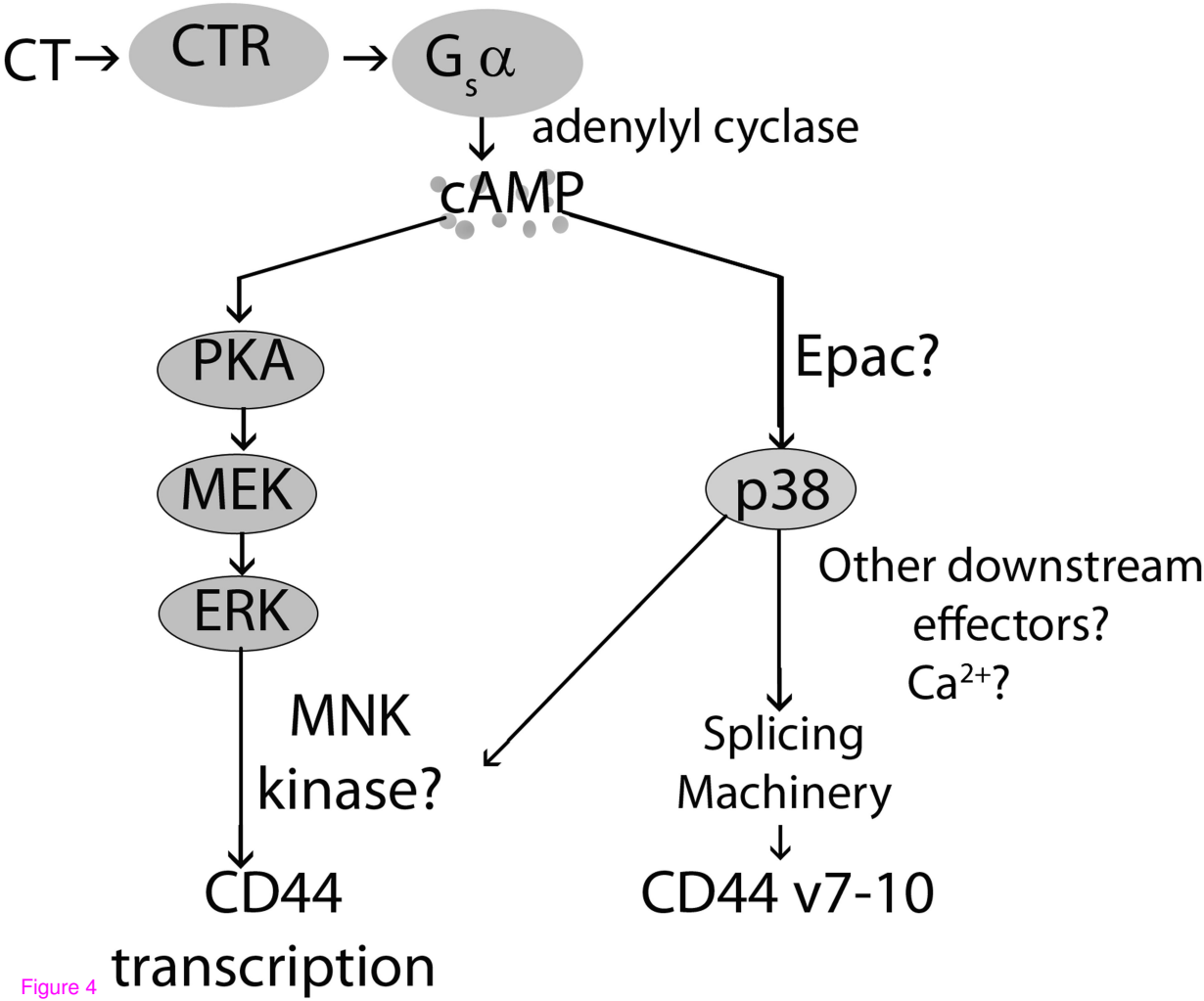
CT→
CTR
→
$G_s\alpha$
adenylyl cyclase
cAMP
PKA
MEK
ERK
Epac?
p38
Other downstream effectors?
Ca^{2+}?
MNK kinase?
Splicing Machinery
CD44 transcription
CD44 v7-10

Figure 4

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Original Article

MicroRNAs 373 and 520c Are Downregulated in Prostate Cancer, Suppress CD44 Translation and Enhance Invasion of Prostate Cancer Cells *in vitro*

Kui Yang, Alina M. Handorean and Kenneth A. Iczkowski

Department of Pathology, University of Colorado Health Science Center, Aurora, Colorado

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Abstract: Prostate cancer (PCa), like most human cancers, features dysregulated CD44 expression. It loses expression of CD44 standard (CD44s), present in benign epithelium, and overexpresses a less abundant splice isoform, CD44v7-10. MicroRNAs 373 and 520c putatively regulate CD44. The levels of these two microRNAs were measured in matched benign and malignant patient tissues and in prostate cell lines. The effects of their transfection on CD44 mRNA and protein were documented. Whether these miRNAs act on CD44 promoter, or its 3' untranslated region (UTR), was studied with luciferase reporter constructs and their influences on migration and invasion were determined in PC-3M cells. *miR-373* and *miR-520c* expression were decreased in PCa cell lines and tissues, in proportion to their decreases in total CD44 mRNA. Exogenous *miR-373* caused a dose-dependent increase in total CD44 RNA, but a decrease in CD44v7-10 RNA, with an optimal dose at 6 nM. At the protein level, however, both microRNAs suppressed CD44. Both migration and invasion were stimulated by *miR-373* and *miR-520c*. The microRNAs had no effect on the CD44 promoter, but did exhibit 3'UTR binding. In conclusion, *miR-373* and *miR-520c* exert their effect in PCa by preventing the translation of CD44 RNA, rather than by degrading the RNA. Despite this observation, they exert pro-invasive functional effects, as previously described in breast cancer cells. Their effects are mediated by binding CD44 3'UTR.
Keywords: MicroRNA, miR-373, miR-520c, prostatic neoplasms, CD44, invasion

Introduction

About 30% of cases of prostate cancer (PCa) undergo a transition from quiescent to aggressive. This transition requires changes in adhesion glycoproteins such as CD44 that allow tumor cells to detach, interact with proteins that digest stromal matrix, and migrate through matrix and intravasate into lymphovascular channels. CD44 is a transmembrane, cell adhesion glycoprotein that mediates cell-cell and cell-stromal interactions, binds hyaluronan and several other matrix substrates, and controls cell shape through the cytoskeleton. CD44 is expressed as a ubiquitous standard (CD44s) isoform, but in epithelial cells, gene products of variant (CD44v) exons get included that lengthen the extracellular portion of CD44. In either isoform, oligomerization at the extracellular domain is required for CD44 function. Global dysregulation of alternate splicing is common in cancer, and in PCa CD44 expression is lost while splicing is altered in favor of a variant isoform which causes invasion [1]. The probable mechanism is that inclusion of abnormal variant sequences alters CD44's ability to oligomerize and its ligand binding [2], potentiating tumor growth, invasion, and metastasis.

CD44, along with 30% of human genes [3], is regulated by at least 851 human microRNAs (miRNAs). Dysregulation of this recently discovered class of noncoding RNAs is also common in PCa [4]. miRNAs that may interact with CD44 have been studied only in breast cancer, not PCa. Recently, Huang *et al* described *miR-373* and *miR-520c*, members of the same miRNA family sharing similar seed sequences [5], as functional oncomiRs in breast cancer [6]. They bound specifically to the CD44 3′ untranslated region (3′UTR) and suppressed CD44; both CD44 knockdown and

miR-373 stimulated tumor invasion and migration. This action can be explained because CD44, particularly CD44s, is a tumor suppressor in breast cancer as it is in PCa. However, CD44 functional implications differ by tumor type: a CD44v confers invasive ability in PCa [1], but CD44s has pro-invasive properties in some tumors such as colon cancer [7]. Thus, we tested whether similar mechanisms and effects were operative in PCa. Because *miR-373* targets the E-cadherin promoter [8] and might also target the CD44 promoter according to our sequence analysis, we explored whether its mode of action was by the promoter or 3′UTR.

Materials and Methods

Cell Lines and Tissues

Benign PrEC and BPH-1, and LNCaP and PC-3 prostate cancer lines and MCF-7 breast cancer cells were from American Type Culture Collection (Manassas, VA). The culture medium for all these cell lines was RPMI 1640 (Invitrogen, Carlsbad, CA) with 10% fetal calf serum. PC-3M cells, a metastasis-derived variant of PC-3, were obtained from Dr. Girish Shah, U. of Louisiana—Monroe. They were grown in RPMI 1640 (Invitrogen) with 12% horse serum, 5% fetal calf serum and antibiotics. Cells were grown in 5% CO_2 incubator at 37ºC. For each experiment, cells in a flask were trypsinized, washed with sterile PBS to remove trypsin, resuspended in basal medium, and counted after dilution with Trypan blue dye using the grid method [2].

Laser Capture Microdissection

We isolated pure benign glandular cells and benign stromal cells. RNA from cryostat sections was prepared using the PicoPure RNA Isolation Kit for frozen tissues (Arcturus, Mountain View, CA) with columns designed to capture short RNA.

Quantitative RT-PCR (qRT-PCR)

Total RNA was prepared from cell pellets using Trizol (Invitrogen) as described by the manufacturer. RNA was further purified by isopropanol precipitation, resuspended in RNase-free water, and its concentration measured. Complementary DNA (cDNA) was synthesized from 4 µg total RNA in 20 µl reaction mixture as described previously [1]. qRT-PCR reactions used 4 µg cDNA plus the manufacturer's master mix and primer/probe sets (Applied Biosystems, Foster City, CA) in a volume of 20 µl.

For miRNA, the qRT-PCR was performed in two different ways for selected samples. In the first (primer + probe) approach, cDNA was prepared with MultiScribe (Applied Biosystems), with a special recombinant Moloney murine leukemia virus (rMoMuLV) reverse transcriptase in an optimal buffer. Unlike mRNAs, miRNAs are not polyadenylated, so hairpin-loop forming primer + probe sets for *miR-373* and *miR-520c* (Applied Biosystems) were used. The cycling conditions for both mRNA and miRNA were: hold 50ºC 2 min, 95ºC 10 min, and then 40 cycles of 95ºC for 15 sec and 60ºC for 1 min, as previously described [9], using the ABI Prism 7500 cycler (Perkin-Elmer, Waltham, MA).

A second, confirmatory qRT-PCR approach used the NCode kit (Invitrogen). After RT, RNA was tailed with poly (A) polymerase, not normally present on miRNA, then a forward primer specific to each miRNA was used together with universal poly-T reverse primer. Detection was done by SYBR green without a probe. Cycling settings were: 50ºC for 2 min, and then 40 cycles of 95ºC for 25 sec and 60ºC for 1 min.

For CD44, samples were run in triplicates with a primer/probe set for all CD44v that brackets the entire variant region [1], one for CD44 total whose probe binds a standard exon, and 18S ribosomal RNA. Primer/probe sets for CD44v were: forward, 5'-AACGCTTCAGCCTACTGCAAA-3'; reverse, 5'-TCTTCCAAGCCTTCATGTGATG-3'; probe, 5'-GATTTGGACAGGACAGGACCTCTTTCAATG-3'. For CD44 total, we used forward, 5'-CAACTCCATCTGTGCAGCAAA-3'; reverse, 5'-GTAACCTCCTGAAGTGCTGCTC-3'; probe, 5'-CATATTGCTTCAATGCTTCAGCTCCACCTG-3'. Primer and probe sets for 18S were proprietary to the manufacturer.

MicroRNA Transfection

A flask of PC-3M cells at 80% confluence was lipofected with *miR-373* or *miR-520c-3p* (IDT, Coralville, IA) or the irrelevant miR Negative Control #2 (Ambion, Austin, TX) for 6 hours using Trans-IT (Mirus, Madison, WI). Cells were allowed at least 24 hours to recover from the

transfection before experiments.

Western Blot Analysis

Cultured cells were directly lysed in their wells using RIPA buffer (Upstate Biologicals, Lake Placid, NY) plus the protease inhibitor mini tablets (Applied Science, Indianapolis, IN). Protein concentration of the cell lysate was estimated by Bradford method. SDS-PAGE was performed on 25 µg sample/lane according to the Laemmli method using the NuPAGE system (Invitrogen, Carlsbad, CA). 5 µl of Kaleidoscope Precision Plus Protein Standards (Bio-Rad) was run in at least one lane. After electrophoresis for 2 hr, the protein was transferred to PVDF. Three primary antibodies were used. To assess CD44v9 (the largest component of the overexpressed CD44v7-10), the membrane was reacted with neat supernatant from the hybridoma cell line HB-258 (ATCC). CD44 total (standard + variant) was assessed using anti-HCAM (DF1485, Santa Cruz Biologicals, Santa Cruz, CA). Anti-β-actin-HRP antibody (Sigma, St. Louis) was used at a dilution of 1:10,000. Membranes were washed 3 x 15 min in TBS with 0.1% Tween 20% and 1:1000 dilution of goat anti-mouse IgG antibody labeled with HRP (Bio-Rad, Hercules, CA) was added in 5% skim milk + TBST for 1 hr. Reactivity was detected using the SuperSignal West Pico Substrate chemiluminescent system (Pierce Biotechnology, Rockford, IL). Each experimental run was conducted at least twice.

CD44 Promoter and 3′UTR Luciferase Constructs

Using the PXP2 plasmid, 1150 bases of CD44 sequence including the start site, and beginning 964 bases upstream to the start site, was cloned in between *Xho* I and *Hind* III sites. Because one of the most effective positive controls to test the promoter responsiveness is *HOXC6* expression [10], we obtained a plasmid to overexpress *HOXC6* in prostate cells (Gift of Dr. Jim Lambert). The overexpression was documented by Western blot analysis using an anti-HoxC6 rabbit polyclonal antibody (Aviva Systems Biology, San Diego, CA) at 1:500 dilution, with goat anti-rabbit secondary antibody at 1:10,000 (Bio-Rad, Hercules, CA), as above. The CD44 3′UTR luciferase reporter plasmid was a gift of Dr. Qihong Huang from Wistar Institute, Philadelphia [6]. Luciferase activity was measured 48 hours after transfection using the firefly luciferase assay (Gold Biotechnologies, St. Louis, MO). The cells were harvested in 20 mM K_2HPO_4 pH 7.8 with 5 mM $MgCl_2$ and 0.5% Triton X-100 buffer for 15 min on ice, and the mixture was centrifuged for 10 min at 4°C. 50 µl of lysate plus 350 µl of luciferase assay buffer as per the manufacturer. The relative luciferase units (RLU) were normalized to protein concentration as determined by Bradford assay.

Invasion and Migration Assays

Invasion was assessed with triplicate 24-well Matrigel two-tier invasion chambers, and migration was assessed with triplicate control inserts, both with 8.0 µm pores (Collaborative Biomedical Products, Bedford, MA) [1, 9]. Cells were seeded at 30,000 per well, untreated or after microRNA transfection. Cells in the upper insert were in serum-free basal medium (RPMI 1640 with 4 mM L-glutamine, 100 µg/mL each of penicillin G and streptomycin). The lower chamber contained chemoattractant medium consisting of 10% fetal bovine serum, 20% conditioned medium from subconfluent culture, and 70% complete medium. The incubation was carried out 24 h in 5% CO_2 incubator at 37°C. The medium from the upper inserts, together with any residual cells were removed off the upper Matrigel surface. The membrane was fixed in methanol and stained with May-Grunwald stain (Sigma, St. Louis, MO) according to manufacturer's protocol. Experiments were repeated twice.

Results

Laser Capture Microdissection

The prior study of *miR-373* and *miR-520c* in breast tissue samples did not distinguish its origin from breast glandular cells or stromal cells [6]. To make this determination in prostate, benign glandular cells and benign stromal cells were microdissected. Taking the $\log_2$ of $-(C_T$ value normalized to β-actin), *miR-373* glandular expression was 0.003377; stromal expression was 0.005048. *miR-520c* was below detection limits in glands and stroma.

Quantitative RT-PCR (qRT-PCR)

To correlate CD44 standard and v7-10

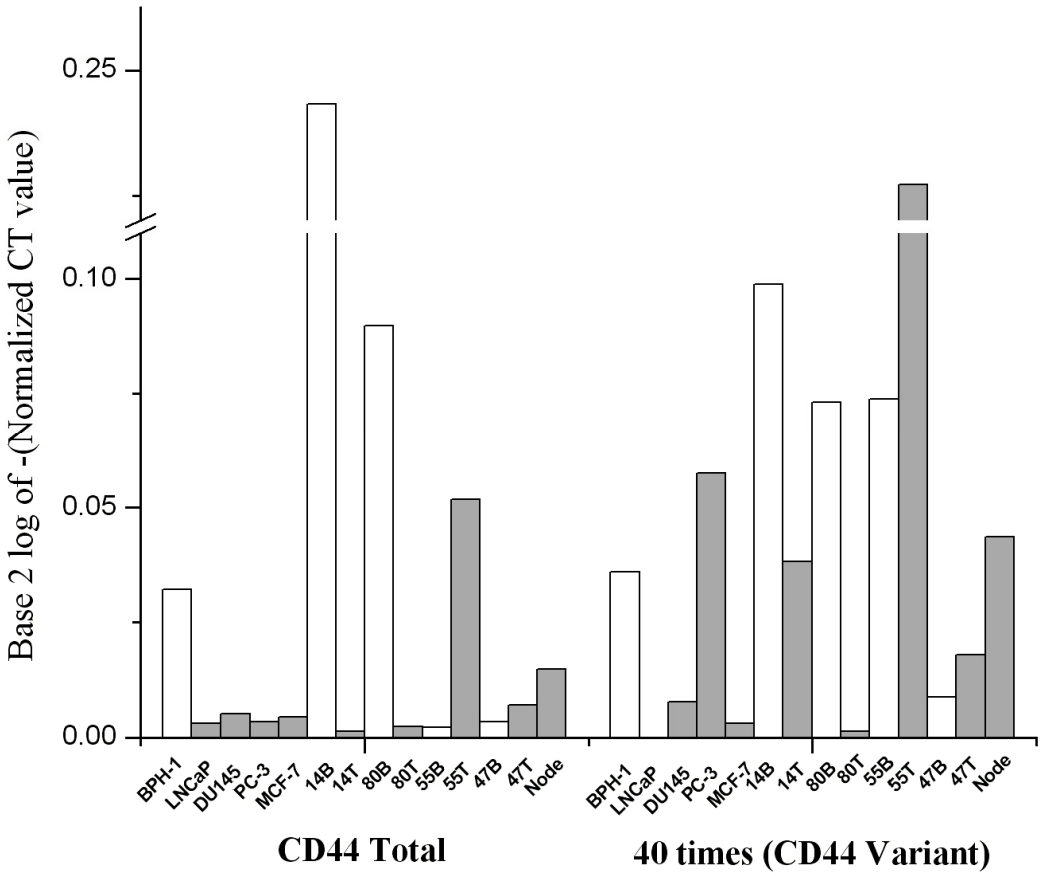

Figure 1 qRT-PCR of CD44 total (left) and variant (right) in 5 cell lines and 5 clinical prostatectomy specimens normalized to 18S RNA. Left: CD44 total, comprising the more abundant CD44s isoform, is higher in benign BPH-1 cell line than in androgen-sensitive LNCaP cancer or androgen-independent DU-145 or PC-3, consistent with previous findings of ours [13] and others [15, 16]. In MCF-7 breast cancer cells, it is low as expected [6]. In matched, frozen section-confirmed, benign (B) and tumor (T) tissues from 4 patients, CD44 is downregulated in the tumor component of 2. In microdissected metastatic prostate cancer from a lymph node (Node), CD44 is low. Right: The less abundant CD44 v7-10 is expressed in androgen-independent prostate cell lines but very low in MCF-7. In 5 tissues, CD44v7-10 is highly expressed in most tumor (T) components and in lymph node with metastasis (Node), as expected from our prior work [1, 12].

expression with miRNA expression in cells and tissues, qRT-PCR was performed. Total CD44 mRNA was lower in all cancer cell lines than benign ones, and in most cancer tissue samples compared with benign tissue from the same patient (**Figure 1**).

By probe + primer method, *miR-373* was highest in the BPH-1 cell line (**Figure 2a**), intermediate in androgen-sensitive, slow-growing LNCaP cells, and very low in androgen-independent PCa as well as in MCF-7, confirming the finding of Huang *et al* who used MCF-7 as a negative control [6]. In matched tissues, *miR-373* was again generally higher in benign tissue than in the tissue sample with cancer (**Figure 2b**).

The differences were greater than could be accounted for merely by *miR-373* being 1.5 times as prevalent in stroma as in glands, and the presence of proportionally less stroma in tumor. These trends were confirmed in separate SYBRgreen qRT-PCR experiments (**Figure 2c**). A similar trend applied to *miR-520c* in cell lines and tissues. *miR-520c* RNA was decreased in primary tumors and metastasis using both a SYBRgreen method (**Figure 2d**) and by using primers with a specific probe (not shown).

Effects on CD44 Promoter and 3′UTR

PC-3M cells were transfected with our CD44 luciferase construct that tests for binding to

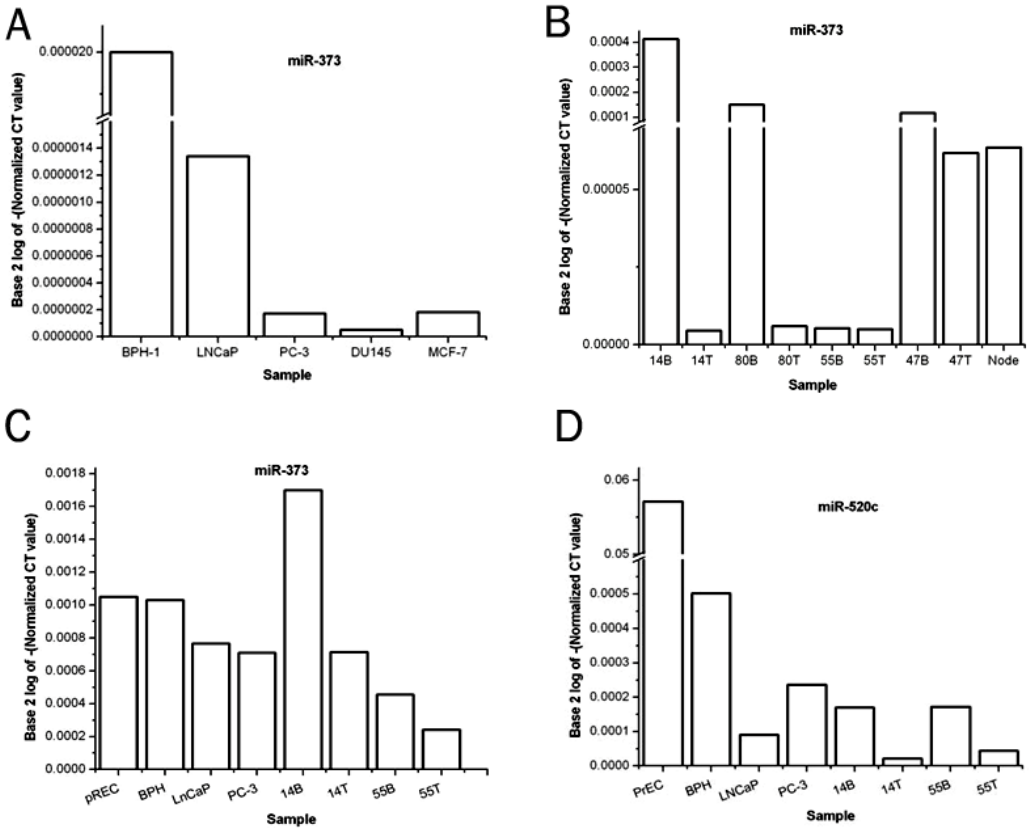

Figure 2 qRT-PCR for *miR-373* by primer and probe method normalized to 18S rRNA (**A** and **B**). **A**. Among cell lines, benign BPH-1 cells have the most *miR-373*, while slow-growing, androgen-dependent LNCaP cancer cells have decreased but detectable *miR-373*. Androgen-independent cell lines PC-3 and DU145 have nearly absent *miR-373*. Level is low in breast cancer cell line MCF-7, consistent with others' findings [5]. **B**. Matched (same patient) tissue specimens. *miR-373* is higher in the benign (B) component than in tumors (T) for 3 of 4 cases. **C**. qRT-PCR by SYBRgreen method for confirmation of *miR-373* expression. Values are higher because they are normalized to β-actin which has lower copy number. *miR-373* is downregulated in cancer cell lines compared to benign PrEC and BPH cells. In 2 patients' tissues, there is downregulation of *miR-373* in the tumor (T) component. **D**. qRT-PCR for *miR-520c* by SYBRgreen method normalized to β-actin. Average expression levels of *miR-520c* were at 1/5 or less the level of *miR-373*. 2 malignant cell lines have less *miR-520c* than 2 benign ones. In 2 patients' tissues, there is downregulation of *miR-520c* in the tumor (T) component.

the promoter region. HOXC6 is documented to regulate the CD44 promoter in PCa cells [10]. We achieved its overexpression by transfection (**Figure 3a**). Cells that were co-transfected with CD44 promoter and HOXC6 demonstrated a strong inhibition of promoter activity by HOXC6 (**Figure 3b**). Overexpression of *miR-373* or *miR-520c* caused ~85% decreases in luminescence readings generated by the 3′UTR construct (**Figure 3c**), signifying that both microRNAs target the CD44 3′UTR.

Effects on CD44 Expression

Transfection of 6 nM synthetic *miR-373* was optimal for detecting its expression (**Figure 4a**). Doses up to 6 nM caused dose-dependent overexpression of CD44 total RNA normalized to untreated cells (using a primer set and probe that detects CD44 total = standard + variants). Thus 6 nM was considered an optimal dose. At the RNA level, divergent effects were noted on total CD44 (stimulation) and on CD44 variant (inhibition, **Figure 4a**). Because miRNA can either degrade mRNA or reversibly inhibit its translation [11], we used western blot analyses to determine whether the directionality of the protein effect was the same. *miR-373* and *miR-520c* both suppressed total CD44 protein compared with untreated positive control or with transfection of an irrelevant, scrambled miRNA (**Figure 4b**).

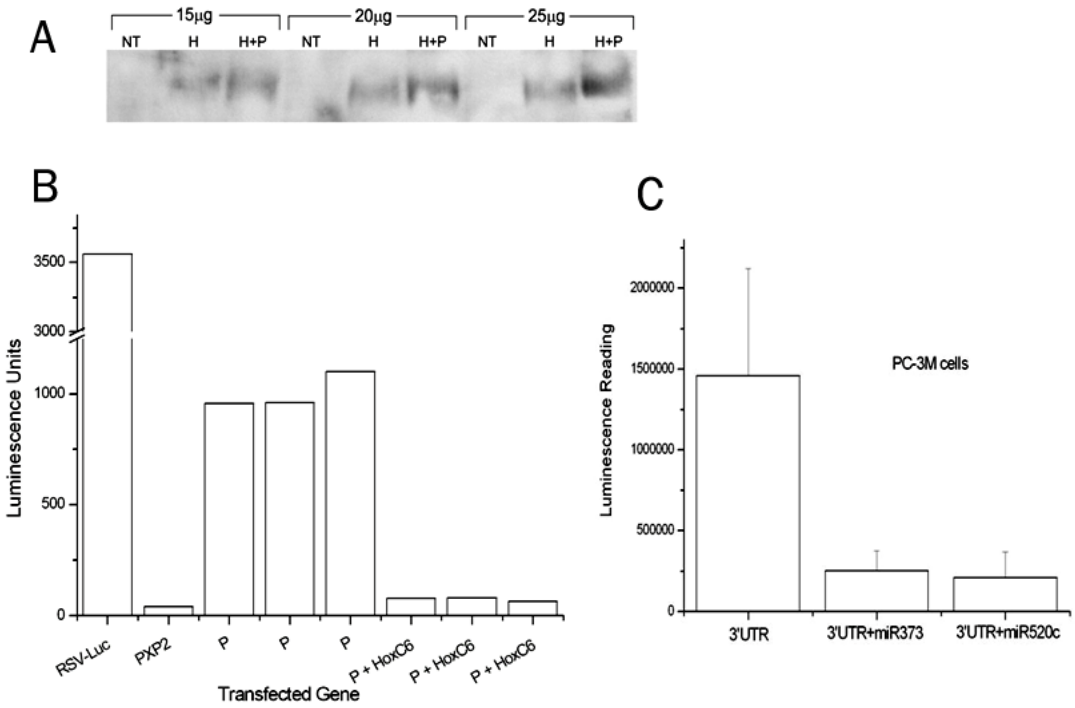

Figure 3 **A.** Successful overexpression of HOXC6 (primary Ab) by Western blot analysis using 15-25 µg protein. PC-3M cells were transfected with either HOXC6 (H) alone or H + CD44 promoter (P), compared to no treatment (NT). **B.** Cells from **A** were subjected to luciferase activity assay for CD44 promoter (P) activity. RSV is the positive control and PXP2 is the negative control. Promoter activity is inhibited by HOXC6. Experiments were performed in triplicates. **C.** Optimized doses of *miR-373* or *miR-520c* were transfected into PC-3M cells subsequent to transfection of a luciferase construct containing the 3′UTR of CD44. Both suppressed gene expression, indicating that CD44 is a direct target of *miR-373* and *miR-520c*.

Effects on Invasion and Migration

Overexpression of *miR-373* increased migrating PC-3M cells more than 5 folds and invading cells more than 3 folds. Moreover, the invaders as a percent of migrators increased from 85% to >100% after *miR-373* transfection. Overexpression of *miR-520c* nearly doubled invasion and migration (**Figure 4c**).

Discussion

Both PCa cells and tumor from prostatectomy show downregulation of *miR-373* and *miR-520c* compared with their benign counterparts. Exogenous doses cause an increase in total CD44 RNA. At the protein level, however, both *miR-373* and *miR-520c* suppress total CD44. This means that *miR-373* and *miR-520c*, like most miRNAs, inhibit, not stimulate, their target; and since CD44 is a tumor suppressor, *miR-373* and *miR-520c* behave as oncomiRs.

The protein finding in our study is concordant with that of Huang *et al* [6], which demonstrated that miRNAs 373 and 520c exerted marked CD44 suppression in MCF-7 breast cancer cells by western blot analysis. Thus, *miR-373* and *miR-520c* function by fundamentally different mechanisms in PC-3M and MCF-7 cells but cause the same phenotypic effect on CD44 protein. The findings of Huang *et al* suggest RNA degradation, whereas our findings suggest the opposite mechanism: RNA accumulation but paradoxically translational repression. It has been noted that mRNA whose repression is mediated by miRNA gets stored in P bodies and conditions of stress can reverse the repression [11]. In hepatoma cells, cationic amino acid transporter 1 (CAT-1) mRNA and reporters bearing the CAT-1 3'UTR or its fragments were able to be relieved from the miRNA *miR-122*-induced inhibition by subjection to different stress conditions [11]. Whether placing prostate cancer cells under

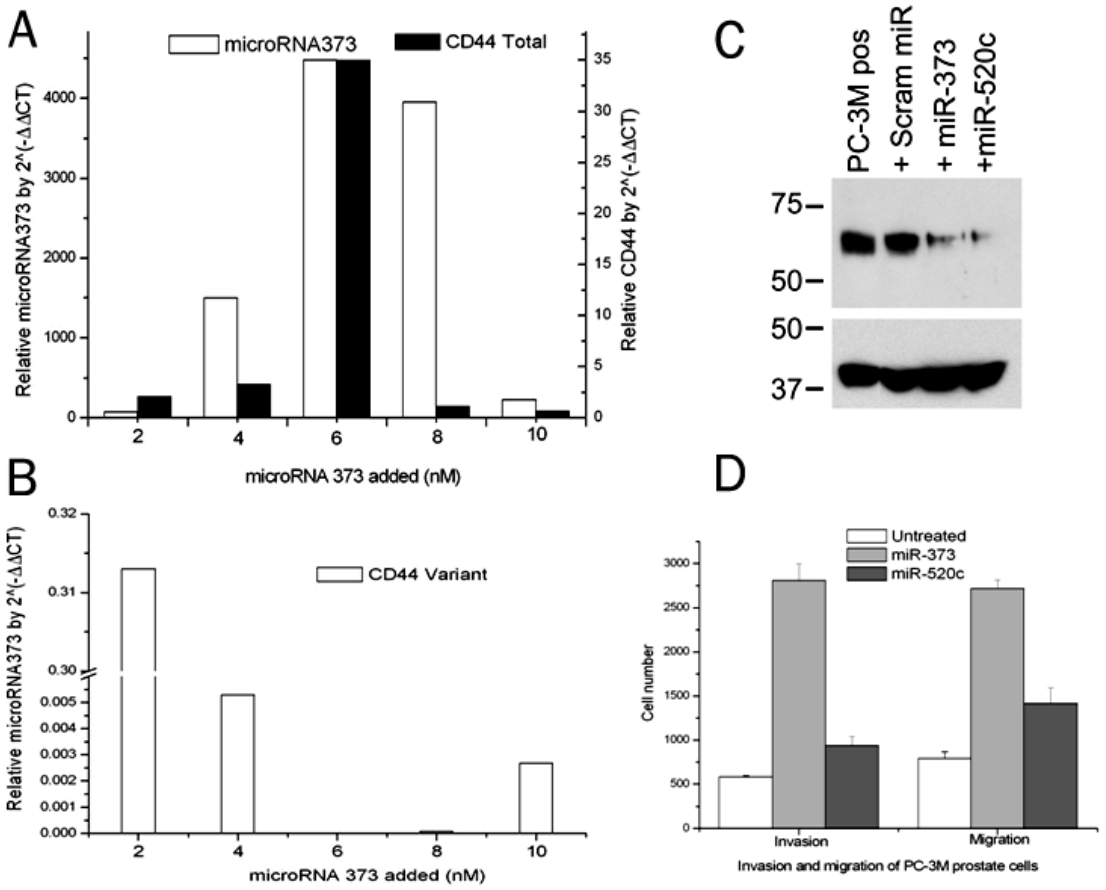

Figure 4 **A.** Left y-axis: Transfection of 6 nM dose of synthetic miR-373 maximized its expression by qRT-PCR. Right y-axis: Doses up to 6 nM caused dose-dependent 35-fold overexpression of CD44 total RNA, normalized to untreated cells (using a primer and probe set that detects CD44 total = standard + variants). **B.** The same dose suppressed CD44v7-10 variant expression. **C.** Top: Western blot analysis using primary antibody for total CD44. Lane 1: PC-3M cells as positive (pos) control, with strong CD44. Lane 2, PC-3M cells transfected with scrambled (+Scram), irrelevant microRNA. Lanes 3 and 4: PC-3M cells transfected with *miR-373* or *miR-520c* respectively, showing CD44 suppression. Bottom: β-actin loading control. **D.** 6 nM dose of exogenous *miR-373* also has functional effects: more than 5-fold increase in migration and more than 3-fold increase in invasion of 30,000 PC-3M cells compared to untreated cells. Migration and invasion almost doubled with *miR-520c*.

stress might affect *miR-373* and *miR-520c* effects on CD44 would be of interest.

Most of CD44 is normally CD44s, a tumor suppressor lost in PCa [2, 13-16]. A curious finding was that *miR-373* stimulated total CD44 RNA but also suppressed the pro-invasive CD44v7-10 variant. This suggests an effect occurring prior to splicing, or a direct effect on splicing. This effect also bespeaks a role of these miRNAs as oncomiRs, like their role in breast cancer, as studied with non-invasive MCF-7 cells and in tissues. In those studies, *miR-373* and *miR-520c* suppressed RNA and protein of the anti-invasive CD44 (total versus variant was not examined), increased migration and invasion, and were upregulated in metastases; expression levels in benign breast were not examined [6]. We have not examined *miR-373* and *miR-520c* in metastases, except for just one case.

Functionally, *miR-373* and *miR-520c* also behave as oncomiRs in PCa despite their downregulation. They stimulated cells to migrate and invade. miRNA downregulation is in fact the trend in PCa, applying to 76 of 85 detectable miRNAs in one study [4]. There are only a few reportedly upregulated miRNAs in PCa. The pro-invasive effect of these miRNAs suggests that, although downregulated in PCa, they target other invasion-promoting genes [6].

We determined by microdissection of whole, benign prostate that at least *miR-373* is present in both glandular and stromal cells of prostate tissue. This distinction was not addressed in breast tissue [6], but suggests that the stromal contribution of miRNA in glandular organs is not negligible. Of course in tumor, the glandular component is much more prevalent, so a lesser stromal contribution might be expected.

Most miRNAs bind a response element in the 3' untranslated region (3'UTR) of targeted mRNA. This either reversibly represses its translation or directs sequence specific degradation [11]. Because some influences on *miR-373* and *miR-520c* and CD44 were fundamentally different from those described before, we examined the possibility that their actions were mediated by the CD44 promoter, using a promoter luciferase construct. We demonstrated that this interaction is through the CD44 3′UTR, again suggesting a unique role in PCa pathophysiology. Certain miRNAs can target complementary DNA promoter sequences to induce gene expression [8]. A recent paper, using a prostate cancer model, identified a *miR-373* binding site in the promoter of E-cadherin [8]. Our sequence analysis also suggested a theoretical *miR-373* binding site in the CD44 promoter; but, we ruled out a promoter site of action of *miR-373* and *miR-520c* in PCa cells. Their effect seems to be exerted only by binding to the 3′UTR.

Our prior work demonstrated a unique role for CD44v7-10 in PCa. By isolating RNA from clinical PCa specimens, we discovered that expression of CD44v7-10 constitutes a unique PCa signature, consistently expressed in both primary PCa and PCa metastatic to other tissues [1, 12, 13]. Interference against CD44v caused a 69% reduction in invasion index compared to untreated control cells [13]. Moreover, PCa loses the splicing ability to produce the standard isoform expressed in benign prostate [1, 14-16]. The discovery of downregulation of *miR-373* and *miR-520c* in PCa is consistent with the dysregulated expression of miRNAs in PCa [3, 17] and the upregulation of the enzyme Dicer [18].

In conclusion, in benign prostate, higher *miR-373* and *miR-520c* correlate with the predominance of CD44s isoform. In prostate cancer, the downregulation of total CD44 and upregulation of its less abundant isoform CD44v correlated with loss of *miR-373* and *miR-520c*. With *miR-373*, an exogenous dose increased CD44 RNA. However, at the protein level *miR-373* and *miR-520c* suppressed total CD44, indicating translational repression. These actions are most likely mediated through the CD44 3′UTR. The loss of CD44 total protein, most of which is CD44s, is probably functionally important in mediating pro-invasive effects of *miR-373* and *miR-520c*. Further work is needed to determine whether these miRNAs prevent or target CD44 splicing factors or splicing.

Acknowledgements

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Please address all correspondences to Kenneth A. Iczkowski, M.D., Pathology-P.O. Box 6511, UCHSC-Campus Mail Stop 8104, Aurora, CO 80010. Tel: 303-724-0155; Fax: 303-724-3712; Email: Kenneth.Iczkowski@UCDenver.edu

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Original Article

Silibinin suppresses cd44 expression in prostate cancer cells

Alina M. Handorean[1], Kui Yang[1], Eric W. Robbins[1], Thomas W. Flaig[2], Kenneth A. Iczkowski[1]

[1]From the Department of Pathology, University of Colorado Health Science Center, Aurora, Colorado; [2]From the Department ofMedicine, Division of Urologic Oncology, University of Colorado Health Science Center, Aurora, Colorado.

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Abstract: Prostate cancer (PCa), like most human cancers, features dysregulated CD44 expression. Expression of CD44 standard (CD44s), present in benign epithelium, is lost in PCa while pro-invasive splice variant isoform CD44v7-10 is overexpressed. The role of CD44 in silibinin's anti-growth effects was uncertain. To assess silibinin's effects on CD44 promoter activity, PC-3M PCa cells were transfected with luciferase-CD44 promoter construct 24 h prior to 25-200 μM silibinin treatment for 48 h. Also, cells' expression of CD44 RNA (by qRT-PCR) and protein (Western blot analysis) was studied. Silibinin was further tested preoperatively on a pilot cohort of 6 men with PCa compared with 7 matched placebo-treated men, with immunostaining for CD44v7-10 in their prostates. In PC-3M cells, silibinin dose-dependently inhibited CD44 promoter activity up to 87%, caused a 90% inhibition of total CD44 and 70% decrease in CD44v7-10 RNA, and at the protein level, decreased total CD44 at 100-200 μM dose and decreased CD44v7-10 after 3 days. Silibinin decreased adhesion to hyaluronan and fibronectin. Silibinin at 100-200 μM inhibited Egr-1, a regulator of CD44 promoter activity. Men treated with silibinin did not differ in tissue CD44v7-10 expression. In conclusion, CD44 inhibition is one mechanism by which silibinin reduces PCa tumorigenicity.

Key Words: Silibinin, prostatic neoplasms; CD44; plasmid; alternate splicing; adhesion

Introduction

About 30% of prostate cancer (PCa) cases undergo transition from quiescent to aggressive, and this transition relies on altered expression of adhesion glycoproteins such as CD44 that allow tumor cells to detach, interact with proteins that digest stromal matrix, migrate through matrix and intravasate into lymphovascular channels. CD44 enables cell-cell and cell-matrix adhesion, primarily to hyaluronan but also to other ligands, and links the cell membrane to the actin cytoskeleton, modulating motility. CD44 is a transmembrane molecule encoded by an alternately spliced gene. It is expressed as ubiquitous, standard (CD44s) isoform but inclusion of one or more of 10 variant (v) exons produces tissue-specific (CD44v) isoforms. Moreover, a unique variant isoform of CD44 is overexpressed in PCa and facilitates PCa cell growth and invasion [1-6].

Silibinin is a nutritional supplement that has gained attention as intervention to prevent or treat cancer. Silibinin is a polyphenolic flavonolignan isolated from milk thistle and artichoke that arrests PCa proliferation *in vitro* [7] with cell cycle arrest in G1 [8], potentiates a chemotherapeutic drugs [9], and *in vivo* increases apoptosis and inhibits angiogenesis [10]. It inhibits cancer invasion by inactivating the PI3K-Akt and MAPK signaling pathways [11] as well as decreasing production of urokinase-plasminogen activator (uPA) and matrix metalloprotease-2 [12]. The early growth response-1 (Egr-1) transcription factor binds to and activates CD44 promoter [13]. Here we investigate these effects of silibinin on CD44 total and variant, adhesion, and Egr-1 expression.

Materials and Methods

Cell lines and treatment

PC-3M cells, a metastasis-derived variant of PC-3, were obtained from Dr. Girish Shah, U. of Louisiana—Monroe. LNCaP and DU145 cells were from ATCC (Manassas, VA). Cells were grown in RPMI 1640 (Invitrogen) with 10% fetal calf serum and antibiotics. Cells were grown in 5% CO2 incubator at 37ºC. For each experiment, cells in a flask were trypsinized, washed with sterile PBS to remove trypsin, resuspended in basal medium, and counted after dilution with Trypan blue dye using the grid method.

Cells were treated with a dose of 25 up to 200 μM silibinin; based on the fact that 100 μM most effectively inhibited invasion, MMP-2, and uPA [11,12]. Vehicle for both agents was DMSO, which was applied to control cells.

CD44 promoter luciferase assays

Using the PXP2 plasmid, 1150 bases of CD44 sequence including the start site, and beginning 964 bases upstream to the start site, was cloned in between Xho I and Hind III sites. The efficacy of the promoter construct had been validated based on its inhibition by HOXC6 [14]. Luciferase activity was measured 48 h after transfection using the firefly luciferase assay (Gold Biotechnologies, St. Louis, MO). The cells were harvested in 20 mM K2HPO4 pH 7.8 with 5 mM MgCl2 and 0.5% Triton X-100 buffer for 15 min on ice, and the mixture was centrifuged for 10 min at 4ºC. 50 μl of lysate were added to 350 μl of luciferase assay buffer as per the manufacturer. The relative luciferase units (RLU) were normalized to protein concentration as determined by Bradford assay.

Real time TaqMan RNA analysis

Total RNA was prepared from cell pellets using Trizol (Invitrogen, Carlsbad, CA) as described by the manufacturer. RNA was further purified by isopropanol precipitation, resuspended in RNAse-free water, and its concentration measured. Complementary DNA (cDNA) was synthesized from 4 μg total RNA in 20 μl reaction mixture as we did previously [15]. At least triplicate samples were run using a primer set that brackets the entire variant region with a probe for CD44v9 [15], primers for CD44 total with a probe that binds a standard exon, and 18S ribosomal RNA. Quantitative PCR reactions were optimized to 4 μg cDNA (0.16 μg with 18S) plus the manufacturer's master mix and primer/probe sets (Applied Biosystems, Foster City, CA) in a volume of 20 μl. The amplification protocol was as follows: hold 50ºC 2 min, 95ºC 10 min, then 40 cycles of (95ºC for 0:15 and 60ºC for 1:00) using the ABI 7500 cycler (Perkin-Elmer, Waltham, MA). Primer/probe sets for CD44v were: forward, AACGCTTCAGCCTACTGCAAA; reverse, TCTTCCAAGCCTTCATGTGATG; probe, GATTTGGACAGGACAGGACCTCTTTCAATG. For CD44 total we used forward, CAACTCCAT CTGTGCAGCAAA; reverse, GTAACCTCCTGAAG TGCTGCTC; probe, CATATTGCTTCAATGCTTCAG CTCCACCTG. Primer and probe sets for 18S were proprietary to the manufacturer.

Western blot analysis

Cultured cells were directly lysed in their wells using RIPA buffer (Upstate Biologicals, Lake Placid, NY) plus the protease inhibitor mini tablets (Roche, Indianapolis, IN). Protein concentration of the cell lysate was estimated by Bradford method. SDS-PAGE was performed on 25 μg sample/lane according to

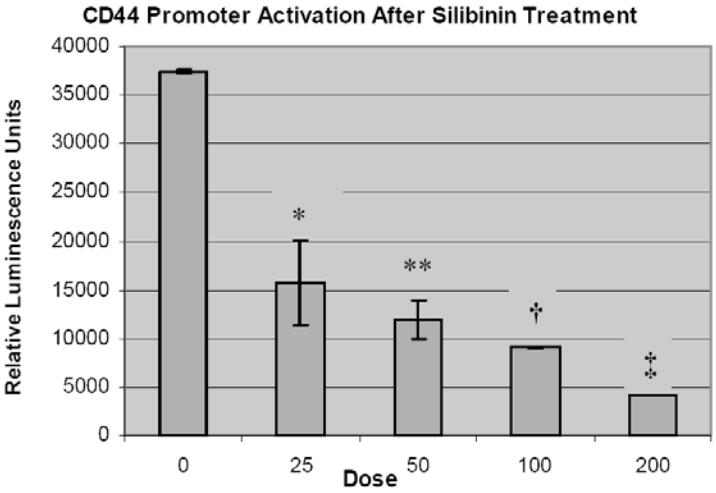

Figure 1. CD44 promoter assays. Transfected PC-3M cells overexpress PXP2 plasmid containing cloned in CD44 promoter + luciferase. Cells were incubated with silibinin for 48 hr after promoter transfection. There is a dose-dependent inhibition of promoter activity. Below: β-actin is shown as a normalizer.

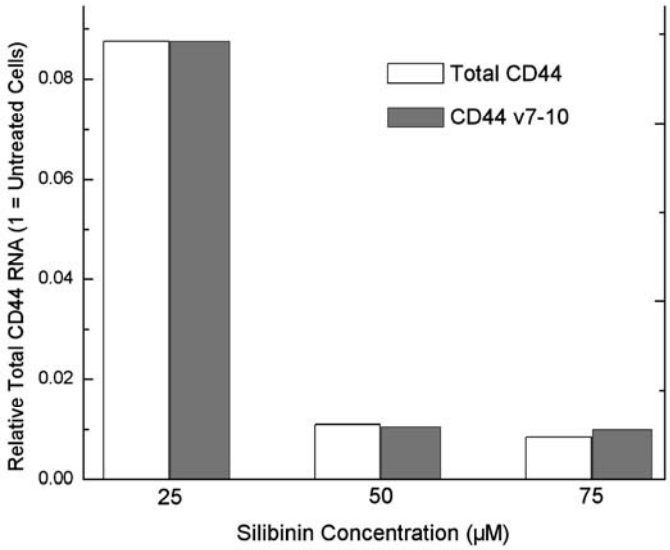

Figure 2. qRT-PCR demonstrates mean CD44 RNA levels after 25-100 μM silibinin treatment, normalized to untreated cells. Total CD44 was 91-99% inhibited. CD44v7-10 was 2.4x control value after 25 μM dose but inhibited at higher doses.

Laemmli method using the NuPAGE system (Invitrogen, Carlsbad, CA). 10 μl of Kaleidoscope Precision Plus Protein Standards (Bio-Rad) was run in at least one lane. After electrophoresis for 2 hr, the protein was transferred to nitrocellulose. Primary antibodies were used. Polyclonal anti-Egr-1 C-terminus (Santa Cruz) was used at 1:200. To assess the overexpressed CD44v7-10 the membrane was reacted with neat supernatant from the hybridoma cell line HB-258 (ATCC). CD44 total (standard + variant) was assessed using monoclonal antibody 156-3C11 (Cell Signaling Technology, Danvers, MA) at 1:1000. Anti-β-actin-HRP antibody (Sigma, St. Louis) was used at a dilution of 1:10,000. Membranes were washed 3 x 15 min in TBS with 0.1% Tween 20% and 1:2000 dilution of goat anti-mouse IgG antibody labeled with HRP (Bio-Rad, Hercules, CA) was added in TBS + 5% skim milk for 1 hr. Reactivity was detected using the SuperSignal West Pico Substrate chemiluminescent system (Pierce Biotechnology, Rockford, IL). Each experimental run was conducted at least twice.

Cell adhesion assays

Cellular adhesion assays as described [15], were carried out using trypsinized confluent untreated, PC-3M cells after 48 hour 100 μM silibinin treatment or vehicle only. Each test condition was set in 8 wells. 96-well black-edged, clear flat bottom Costar plates (Cole-Parmer, Vernon Hills, IL) were coated with 20 μg/ml fibronectin (Becton Dickinson, Bedford, MA) or 2 mg/ml hyaluronan (Sigma, St. Louis, MO) [15] at 37°C overnight. To measure baseline nonspecific binding, other wells were coated with 1 mg/ml BSA. 1x106 cells suspended in 1 mL PBS were incubated with the dye BCECF-AM (Dojindo, Tokyo) for 15 minutes at 37°C [16]. After two washes of the cells with PBS, cells were added to plates at a density of 3x104 per well and incubated at 37°C for 90 min. Fluorescence intensities at 530 nm were measured using a Bio-Tek FL-600 fluorescent plate reader. Nonadherent cells were removed with successive PBS washes, reading fluorescence intensities with PBS in the wells after each. Adhesion was calculated [15] as % cells bound=(100) fluorescence intensity after second wash / fluorescence intensity of total cells plated. The assay was repeated twice.

Immunostaining for CD44v9 in prostate cancer patients

Under written informed consent, six men planning to undergo radical prostatectomy received 13 g silibinin-phytosome daily in 3 divided doses for a 2-4 week duration, and 7 men were untreated controls. Plasma levels of silibinin peaked at 25-150 μM with a half-life ranging from 2-5 hours [17]. The primary antibody was neat supernatant from the hybridoma cell line HB-258 (ATCC) secreting mouse anti-human antibody to CD44v9. The secondary antibody was goat anti-mouse IgG antibody labeled with HRP (Bio-Rad, Hercules, CA).

Statistical analysis

CD44 promoter activity data were analyzed by ANOVA and post t-test, comparing activity at various doses. TaqMan data were analyzed by the 2(-ΔΔCT) method [18] to determine fold change in gene expression (untreated

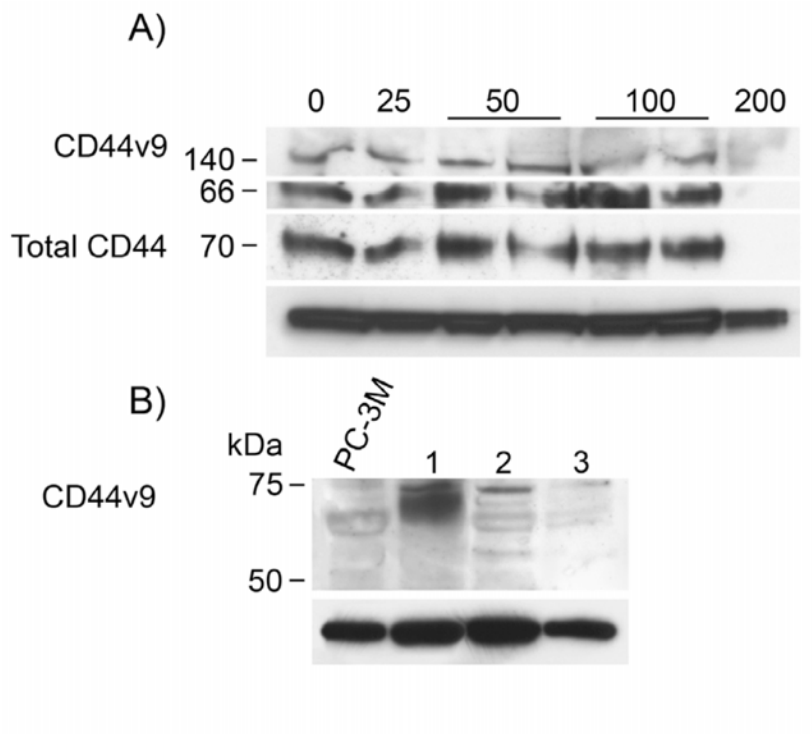

Figure 3. A) Western blot analysis of 25-200 μM silibinin-treated PC-3M cells at 24 h, using monoclonal anti-CD44v9. Predominant band is 66kDa, characteristic for CD44 variant cleavage product [3] although there is some full-length protein at 140 kDa. Monoclonal anti-total CD44, predominantly CD44s, is also detected. At 24 hr, total CD44 and CD44v9 are decreased only at 200 μM dose. Below: β-actin is shown as a normalizer. B) Extending the silibinin exposure of cells to 3 days caused a most marked inhibitory effect on CD44v9.

cells=1.00). The ΔCT was taken as the difference between the CD44v or CD44 total and the 18S ribosomal RNA CTs. The ΔΔCT was obtained using the mean ΔCT of untreated cells as calibrator. Each TaqMan result was compared to 1.00 using 2-tailed paired t-test. Statistical significance was set at $p < 0.05$. Adhesion assay data are expressed as mean ±SD. The significance of differences among group means was tested by 2-tailed paired t-test.

Results

Silibinin suppresses CD44 promoter activity, RNA and protein

Transfection of our CD44 promoter construct into PC-3M prostate cancer cells for 24 h was followed by silibinin treatment for 48 h to assess the effects of these agents. Silibinin significantly dose-dependently inhibited promoter activity, up to 86.7% ($p<0.001$) at 200 μM (**Figure 1**). Doses from 25 μM to 200 μM differed significantly from no treatment; and a dose of 200 μM caused significantly less promoter activity than all lower doses. Mean RNA levels after all doses of silibinin treatment, normalized to untreated cells, were more than 90% lower for total CD44. CD44v7-10 was noted to be increased at 25 μM dose but decreased at higher doses (**Figure 2**). At the protein level, silibinin's effect at the protein level was minimal after 1 day, with western blot analysis (**Figure 3**) demonstrating inhibition of total CD44 and CD44 variant mainly at the highest, 200 μM dose. We then performed a time course experiment, however, and noted that the earlier CD44v7-10 stimulation turned to a strong inhibition at 3 days, consistent with silibinin's inhibition of CD44v7-10 RNA.

Adhesion assays

Compared to untreated cells, PC-3M cells after 100 μM silibinin had a 66% reduction in rate of adhesion to hyaluronan and 47% reduction in adhesion to fibronectin (**Figure 4**).

Clinical treatment with silibinin

Prostate tissues from 6 men with prostate cancer treated with silibinin and 7 treated with placebo were immunostained for the functionally important CD44 v7-10 using anti-CD44v9, directed against the largest portion of the included protein product CD44v7-10. Immunoreactivity was scored on a scale of 0-3+. The mean reactivity in treated and untreated men respectively were 1.0 and 1.3 for cancer, 2.9 and 2.6 for benign basal cells, 0.7 and 0.8 for benign secretory cells, and 0.8 and 0.7 for atrophic glands (p=NS). No

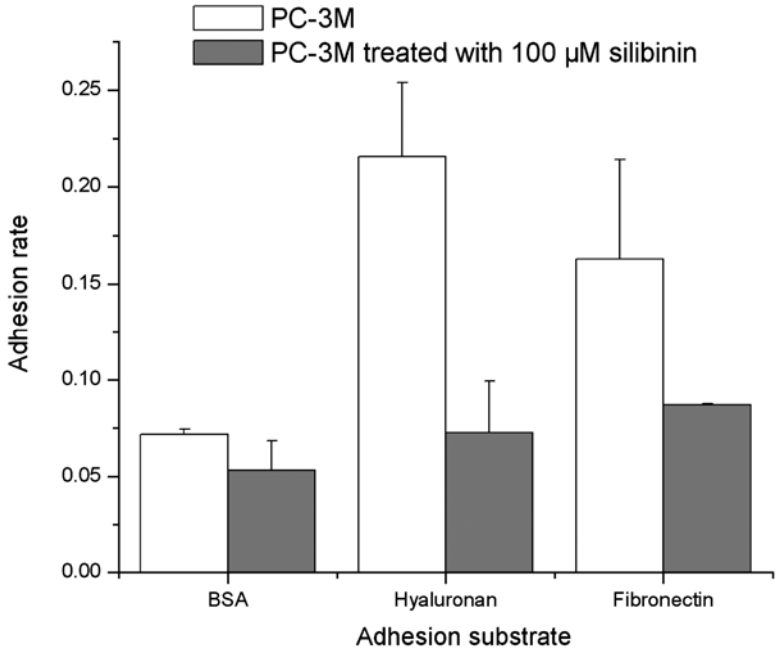

Figure 4. Adhesion assay with PC-3M cells after 100 μM silibinin treatment. Adhesion to both hyaluronan and fibronectin is diminished to baseline level, near that of BSA-coated wells, after silibinin treatment.

significant differences were noted.

Candidate mediators of silibinin effect

Silibinin dose of 100 or 200 μM inhibited Egr-1 expression (**Figure 5**). Bands were observed at 55 kD consistent with the theoretical monomeric form of Egr-1, with dimeric form bands near 110 kD [19]. 100 μM concentration equals the plasma level attained in patients [17]. As a negative control, slow growing, androgen-sensitive LNCaP cells were negative, while more aggressive DU145 cells expressed Egr-1. Immunoblots against EGFR and phospho-EGFR disclosed no silibinin effect (data not shown).

Discussion

We discovered that the nutrient silibinin reduces CD44 promoter activity, resulting in decreased total CD44 RNA. Inhibition of CD44v7-10 was less marked; however, at the protein level, this inhibition was strongly evident after 3 days of treatment. Our interest in CD44v7-10 variant isoform expression in PCa extends back several years. By isolating RNA from clinical PCa specimens, we found CD44v7-10 to be a unique PCa signature, consistently overexpressed in androgen-independent cells such as PC-3, primary PCa, and PCa metastatic to other tissues [1-3]. Interference against CD44v7-10 reduced invasion index by 69% compared to untreated control cells [3]. Moreover, PCa loses the splicing ability to produce the standard isoform expressed in benign prostate that constitutes most of total CD44 [3-6] along with moderate levels of CD44v3-10 variant [6]. In PC-3 prostate tumor, CD44s transfection reduced invasion *in vitro* [20] and its tumorigenicity in mouse xenografts [21]. Silibinin's ability to reduce pro-invasive CD44v7-10 undoubtedly contributes to its effects.

CD44's adhesive affinities depend on its ability to cluster and multimerize, which is altered by a change in CD44s to CD44 variant ratio. Thus we tested PCa cell adhesion to hyaluronan and fibronectin, two ligands that are most functionally important for CD44 in PCa cells. Binding to these two ligands was most strongly altered in PCa cells compared to benign BPH-1 cells [20], which bound hyaluronan strongly and fibronectin

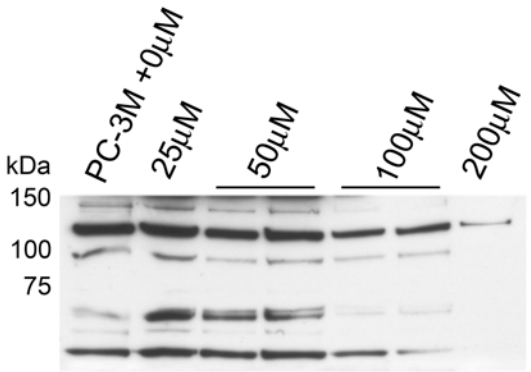

Figure 5. Western blot analysis of 25-200 μM silibinin-treated PC-3M cells using polyclonal anti-Egr-1. LNCaP and DU145 whole cell lysates are used as negative controls. Monomeric 55-kD and dimeric 110-kD cytoplasmic Egr-1 bands are seen, as well as a 75-kD band that had been described as specific to nuclear Egr-1 [19].

minimally. The androgen-sensitive LNCaP cells bound both ligands equally, while PC-3 and their derivatives bound most strongly to fibronectin, probably representing an integrin-independent mechanism of binding to fibronectin. Moreover, re-expression of CD44s significantly increased adhesion to hyaluronan and decreased adhesion to fibronectin. Here, we observed a loss of total CD44 (which is largely CD44s) after silibinin treatment, consistent with our decreased hyaluronan binding, and lower CD44v7-10 which would mediate less fibronectin binding. Because of the decreased hyaluronan binding, silibinin does not strictly cause reversion to the benign phenotype but is consistent with the decreased migration [7], invasion [11,12] and possibly decreased *in vivo* growth [8] of cancer cells after silibinin treatment. The only prior study of silibinin's effects on PCa cell adhesion were done with type I collagen, showing inhibited adhesion of PC-3 cells to that ligand [7].

By immunostaining, prostatectomy tissue from silibinin-treated men did not differ in CD44v9 expression from that of untreated men. This lack of effect has two good explanations. First, the treatment was only for 2-4 weeks, minimizing the opportunity for the agent to alter CD44 expression in the tumor. Second, samples from this cohort of men are being tested to determine the tissue absorption into the prostate. Despite the high serum levels achieved, we have not confirmed reliable tissue penetration.

Silibinin's suppression of CD44 promoter activity which diminishes total CD44 RNA and protein is probably indirect. Factors that regulate CD44 expression by binding its promoter include Egr-1 [13,22], epidermal growth factor (EGF) [23], activating protein-1 (AP-1) [24], and H-ras [25]. Early growth response (EGR) genes encode zinc finger DNA-binding nuclear transcription factors, control cell proliferation, and show divergent expression in various human tumors. In PCa, Egr-1 binds to the androgen receptor [26] with important physiologic implications. One of us [23] demonstrated increased Egr-1, but not Egr-2 or EGR-α expression, in malignant prostate tissue as compared with weak expression in benign tissue. By in situ hybridization, the expression of Egr-1 was highest in tumors of Gleason score 8-10 than in those of lower Gleason score. Here we showed for the first time that the therapeutically relevant 100 μM dose of silibinin suppresses the high level of Egr-1 in prostate cancer (PCa) cells, suggesting that Egr-1 modulates, at least in part, the effects of silibinin on CD44.

Our findings are not meant to imply that Egr-1 is the sole mediator of silibinin's effects on CD44. Silibinin was reported to act on the MAP kinase pathway [11]. We have shown that MEK and p38 components of the MAP kinase pathway are functionally important for PCa cell expression of CD44 as well as possibly in its splicing [27]. Silibinin also inhibits the PI3K-Akt signaling pathway [11]. Further, it is likely that Egr-1, by binding to GC-rich motifs of other genes, exerts pro-growth effects relevant to PCa that are not dependent on CD44. These include activation of ICAM-1/CD54 [13], which is relevant to PCa growth [28], repression of Fas/CD95 gene [13], required for apoptosis, and repression of transforming growth factor-β Type II receptor, shutting off a growth-inhibitory pathway [29].

In conclusion, silibinin exerts functional effects on CD44 through the CD44 promoter, leading to reduced CD44 total and CD44v7-10 expression. These changes are accompanied by altered adhesion to known extracellular matrix substrates of CD44.

Acknowledgement

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Please address correspondences to: Kenneth A. Iczkowski, M.D., Pathology—P.O. Box 6511, UCHSC—Campus Mail Stop 8104, Aurora, CO 80010. Tel: 303-724-0155, Fax: 303-724-3712, E-mail: Kenneth.Iczkowski@UCDenver.edu

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VIRUS-MEDIATED ALTERATIONS OF CD44 ISOFORM EXPRESSION IN PROSTATE CANCER CELLS DECREASE GROWTH AND INVASION, INCREASE CHEMOTHERAPY SENSITIVITY, AND ALTER LIGAND ADHESION

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Kui Yang, Ph.D.[1]

?Eric W. Robbins, M.S.[1]

Kenneth A. Iczkowski, M.D.[1]

[1]From the Department of Pathology, University of Colorado Health Science Center, Aurora, Colorado.

Address correspondence to Kenneth A. Iczkowski, M.D., Pathology—P.O. Box 6511, UCHSC—Campus Mail Stop 8104, Aurora, CO 80010. Tel: 303-724-0155, Fax: 303-724-3712, E-mail: Kenneth.Iczkowski@UCDenver.edu

Abstract

Background. Prostate cancer (PCa) like most human cancers, features dysregulated CD44 expression. PCa loses expression of CD44 standard (CD44s), present in benign epithelium, and overexpresses a novel splice variant isoform, CD44v7-10.

Methods. Using viral gene delivery to PC-3, PC-3M, and Dunning rat PCa cells, we enforced CD44s re-expression, RNAi against CD44v7-10, [or both?). Proliferation, anchorage-independent growth, migration and invasion, adhesion, and Docetaxel sensitivity assays were carried out. [Merlin stuff?.Mice?]

Results. In PC-3M cells both these alterations of CD44 expression reduced proliferation, invasion, and soft agar colony formation. PC-3 cells re-expressing CD44s had increased Docetaxel sensitivity. In Dunning cells, reliable alterations of CD44 could not be achieved.

Conclusion. Stable re-expression of CD44s and silencing of CD44v7-10 show good potential for PCa gene therapy.

Keywords: prostatic neoplasms; CD44; alternate splicing; invasion; lentivirus

1. Introduction

About 30% of cases of prostate cancer (PC) undergo transition from quiescent to aggressive. In this transition, there is altered expression of adhesion glycoproteins such as CD44 that allow tumor cells to detach, interact with proteins that digest stromal matrix, and migrate through matrix and intravasate into lymphovascular channels. CD44 is a transmembrane molecule encoded by an alternately spliced gene. It is expressed as a ubiquitous, standard (**CD44s**) isoform, but inclusion of one or more of 10 variant (**v**) exons produces tissue-specific (**CD44v**) isoforms. CD44 is involved in multiple cellular functions. It enables cell-cell adhesion, binds hyaluronan and other matrix ligands, and links the cell's membrane to its actin cytoskeleton, modulating motility.

By isolating RNA from clinical PC specimens, we discovered that expression of CD44v7-10 variant isoform constitutes a unique PC signature, consistently expressed in both primary PC and PC metastatic to other tissues [1-3]. Interference against CD44v caused a 69% reduction in invasion index compared to untreated control cells [3] and altered the ligand binding affinities [4]. Moreover, PC loses the splicing ability to produce the standard isoform expressed in benign prostate [3, 5, 6] and an epithelial CD44v variant [7]. In PC-3 prostate tumor, CD44s transfection into PC-3 reduced growth *in vitro* and tumorigenicity in mouse xenografts [8].

In prior work, we enacted only transient CD44v RNAi [3] using the vector U6pBS which lacks a drug-resistance selection gene, and later, a vector with GFP signal [4,9]. Now, we have cloned into lentivirus a CD44v7-10 RNAi sequence and two CD44s re-expression sequences—one expressing CD44s as a fusion protein with luciferase at the cytoplasmic C-terminus, and the other expressing these as separate proteins. Since this stable transfection should provide functional results that are most reliable, here we assess the potential of altering CD44 expression for human gene therapy with a number of in vitro tests. Because the two re-expression transfectants differ in the freedom of their cytoplasmic tail, we postulated that merlin, a binding partner of CD44 tail, would interact differently with them.

2. Materials and Methods

2.1 Cell lines

PC-3 prostate cancer cells and 293T cells were from American Type Culture Collection (Manasas, VA). PC-3M cells, a metastasis-derived variant of PC-3, were from Dr. I. J. Fidler, M.D. Anderson Cancer Center, Houston, TX. A metastasizing strain of Dunning rat prostate cells, chosen for the ability of subcutaneous grafts to metastasize to lungs [10,11](Gao 1997, 1998) was a gift from Dr. Allen Gao, University of California – Irvine. The culture medium for PC-3M and Dunning cells was RPMI 1640 (Invitrogen, Carlsbad, CA) with 10% fetal calf serum (FCS) and antibiotics. Medium for Dunning cells was supplemented with 250 nM dexamethasone (Sigma, St. Louis, MO). PC-3 cells were grown in F12-K medium with FCS. 293T cells were in Dulbecco's modified Eagle medium with FCS. Cells were grown in 5% CO_2 incubator at 37C. For each experiment cells in a flask were trypsinized, medium with serum was added to neutralize trypsin, and cells were diluted with Trypan blue and counted by grid method [4].

{2.2 Plasmid (Transient) CD44 Transfectants

A flask of PC-3M cells at 80% confluence was treated with DNA-lipid complexes formed from 10 µg pTracer-CD44s re-expression [7], or CD44v7-10 RNAi plasmid [3] and 10 µl of Metafectene (Biontex, Germany) in 3 ml of complete medium. After 24 hours, with 20% of cells GFP-Positive, medium was changed to 8 ml fresh complete medium. Blasticidin-resistant transfectants were further selected by adding 250 µl of blasticidin, yielding >50% GFP+ cells. DELETE?}

2.2 Viral (Stable) CD44 Transfectants and Merlin Transfectants

Three constructs were made in Lentivector pLEX-MCS (Open Biosystems, Huntsville, AL), namely: Lenti-luciferase, Lenti-CD44s-luciferase, and Lenti-CD44s-RSV-luciferase (**Figure 1**). Lentivector contains the cytomegalovirus promoter, associated sequences, and puromycin resistance gene. Lenti-luciferase was made by inserting the luciferase fragment from a plasmid (gift of Dr. J. Shen, Department of Radiation Oncology, Univ. of Colorado Denver) behind CMV promoter. A "fusion" construct, Lenti-CD44s-luciferase, was then made by PCR-amplifying the CD44s fragment from our

pTracer construct [7] and subcloning it into Lenti-luciferase. The fusion protein expressed comprises CD44s with luciferase at the C-terminal end. The "separate" construct Lenti-CD44s-RSV-luciferase was made by inserting an RSV promoter between CD44s and luciferase sequences to drive luciferase expression. This construct expresses separate CD44s and luciferase proteins.

Four shRNA constructs were made for knocking down CD44 variant 9 (of CD44v7-10). Three sequences were generated by Extractor 4 computer program, and the fourth was from our prior publication [3]. Retrovirus was produced from pSuper-RETRO (OligoEngine, Seattle) derived from pSuper [~~10~~12].

$2x10^6$ 293T cells were plated per 10 cm dish, and grown to 80% confluence in complete growth medium the next day. For CD44s, 293T cells were transfected with Lentivector, packaging plasmid (psPAX2), and envelope plasmid (pMD2.G) using 3:1 PEI:DNA. For RNAi against CD44v7-10, the procedure was identical to the above, except the 3 packaging vectors were pJK3, pTAT2, and pVSVG. After 48 h, supernatant was harvested and filtered with a 0.45 µm syringe filter. Incubation with viral supernatant for 2 days was used to infect the PC-3, PC-3M, and Dunning (metastasizing) cell lines. Cells were passaged into a 75 cm^2 flask and supplied with puromycin to final concentration of 1 µg/ml. A stable cell line was maintained by changing this selection medium every 3-7 days.

Plasmid pUHD10-3 that contained *Eco*R1 fragments for either wild-type merlin cDNA or ineffective, mutant merlin, was a gift of Dr. D. H. Gutmann of Washington University in St. Louis [~~11~~13]. PCa cells were transfected using PEI in the same manner as the 293T cells.

2.3 Real Time Quantitative RT-PCR

To confirm altered CD44 expression, a primer + probe set that detects CD44s, or a set that detects CD44v7-10 (Applied Biosystems, Foster City, CA) was used as described [~~12~~14]. Detection of 18S ribosomal RNA was done simultaneously as a normalizer.

2.4 Migration and invasion assays

We used a population of PC-3M cells after plasmid transfection with at least 50% GFP-positive cells, transfected with RNAi against CD44v7-10, or PC-3M cells stably infected with lentivirus (for CD44s) or pSUPER-derived retrovirus (for CD44v RNAi). Invasion was assessed with triplicate 24-well Matrigel two-tier invasion chambers, and migration was assessed with control inserts, both with 8.0 µm pore diameter (Collaborative Biomedical Products, Bedford, MA) [4]. Untreated cells or those expressing a construct were seeded at 30,000[15,000] per well. Cells in the upper insert were in serum-free basal medium (RPMI 1640 with 0.1% BSA, 4 mM L-glutamine, 100 µg/mL each of penicillin G and streptomycin). The lower chamber contained chemoattractant medium consisting of 10% fetal bovine serum, 20% conditioned medium from subconfluent culture, and 70% complete medium. The incubation was carried out 24 h in 5% CO_2 incubator at 37C. The medium from the upper inserts, together with non-invading cells were removed off the upper Matrigel surface. The lower surface was fixed in methanol and stained with May-Grunwald stain (Sigma~~,~~) according to manufacturer's protocol.

2.5 Soft agar colony formation and growth assays

A bottom layer was formed using 2 ml of complete medium with 1% agarose that was poured into each well of 6-well plates and solidified at 4ºC. PC-3M cells with or without viral infection, 5000/well, were mixed in complete medium with 0.5% agarose and seeded as a top layer. The agarose was solidified at 4ºC and then incubated at 37ºC. On day 14, the colonies were stained with 1 ml of PBS containing 0.5 mg/ml of p-iodonitrotetrazolium violet (Sigma, St. Louis, MO). Only live cells convert this into a colored product. Colonies were counted in 20 microscopic fields of 100x. For growth assays, 30,000 cells per 6-well plate well were seeded and were harvested and counted 4 days later by grid method.

2.6 Cellular adhesion assays

Assays were carried out as described [~~13~~15] using trypsinized confluent untreated or virally treated cells. Each test condition was set in 8 wells and each experiment repeated twice. 96-well black-edged clear flat bottom Costar plates (Cole-Parmer, Vernon Hills, IL) were coated with optimal concentrations of ligands [~~13~~15], using 8 wells to test each one, at 37ºC overnight. As controls, 8 wells were coated with 1 mg/ml BSA to measure baseline nonspecific binding. $1x10^6$ cells suspended in 1 ml

PBS were incubated with the dye BCECF-AM (Dojindo, Tokyo) for 15 minutes at 37ºC. After two washes of the cells with PBS, cells were added to plates at a density of $5x10^4$ per well and incubated at 37ºC for 90 min. Fluorescence intensities at 530 nm were measured using a Bio-Tek FL-600 fluorescent plate reader. Nonadherent cells were removed with 2 PBS washes. Fluorescence intensities with PBS in the wells were measured. Adhesion was calculated [~~13~~15] as % cells bound=(100) fluorescence intensity post-wash / fluorescence intensity of total cells plated. Data are expressed as mean ±SD. The significance of differences among group means was tested by two-tailed Student *t*-test.

2.6 Western blot analysis

Cultured cells were directly lysed in their wells using RIPA buffer (Upstate Biologicals, Lake Placid, NY) plus the protease inhibitor mini tablets (Applied Science, Indianapolis, IN). Protein concentration of the cell lysate was estimated by Bradford method. SDS-PAGE was performed on 25 µg sample/lane according to the Laemmli method [~~14~~16] using the NuPAGE system (Invitrogen, Carlsbad, CA). 10 µl of Kaleidoscope protein marker (Bio-Rad, Hercules, CA) was run in at least one lane. After electrophoresis for 2 hr, the protein was transferred to PVDF. Three primary antibodies were used: to assess CD44v9 (the largest component of the overexpressed CD44v7-10) the membrane was reacted with neat supernatant from the hybridoma cell line HB-258 (ATCC). CD44 total (standard + variant) was assessed using anti-HCAM (DF1485, Santa Cruz Biologicals, Santa Cruz, CA). Anti-β-actin antibody (Sigma, St. Louis) was used at a dilution of 1:10,000. Membranes were washed 3 x 15 min in PBS with 0.5%% Tween-20 and 1:1000 dilution of goat anti-mouse IgG antibody labeled with biotin (Bio-Rad) was added at 1:9000 dilution in 5% skim milk for 1 hr. Reactivity was detected using a chemiluminescent system (SuperSignal West Pico Substrate, Pierce Biotechnology, Rockford, IL). Each experimental run was conducted at least twice.

2.7 Docetaxel Sensitivity Assays

Cells were seeded on a 96-well plate containing 5000 cells/well. Docetaxel (Taxotere, Sanofi Aventis, Bridgewater, NJ) was administered to wells at doses from 0-100 nM. After 24 hours, MTS assay (CellTiter 96AQ, Promega, Madison, WI) for proliferation was performed.

2.8 Statistical Analysis

TaqMan data were analyzed by the $2(-\Delta\Delta C_T)$ method [~~15~~17] to determine fold change in gene expression (untreated cells=1.00). The ΔC_T was taken as the difference between the CD44v or CD44 total and the 18S ribosomal RNA C_Ts. The $\Delta\Delta C_T$ was obtained using the mean ΔC_T of untreated cells as calibrator. Each TaqMan result was compared to 1.00 using 2-tailed paired t-test. Statistical significance was set at $p < 0.05$.

3. Results

3.1 Establishment of altered CD44 expression

Western blot analysis (**Figure 2**) for total human CD44 in PC-3M luciferase-positive cells documented re-expression of CD44s at 85 kD, in the cell lines bearing the construct for a fusion or [?] separate protein. Western blot analysis for CD44 variant demonstrated successful knockout with one of four viral constructs (the 1522 cells but not the 1519). CD44s re-expression as a fusion or separate protein did not affect CD44 variant. In the Dunning cells with luciferase signal, re-expression of human CD44s as a fusion or separate protein could not be documented on western blot analysis, and human CD44v was absent.

The morphology of PC-3M cells re-expressing CD44s as a separate or fusion protein (**Figure 3**) was much more rounded than PC-3M expressing luciferase only. By Real Time RT-PCR, we achieved 6.58-fold re-expression of CD44s in cells expressing the "separate" protein [need to repeat for "fusion"]. The 1522 RNAi construct had 90.4% silencing of CD44v7-10; the variant was also absent by western blot analysis, and this transfectant was used for further study.

3.2 Migration and invasion

In PC-3M cells, expression of the CD44s as a separate protein, [as a fusion protein with luciferase? –need to repeat], caused significant decreases in both migration and invasion compared with luciferase-only control (**Figure 4**). This pattern held true for Dunning cells as well, although based on western blot

results above, its specificity is questioned. Performing RNAi against human CD44v9 inhibited migration and invasion of PC-3M but not Dunning cells, possibly because a few bases are different between human and rat (Hoffmann).

3.3 Attached cell growth and soft agar colony formation

The CD44s-separate protein PC-3 cells had a 35% decrease in attached cell growth, unlike CD44s-fusion cells (**Figure 5a**). Dunning cells: decreased for fusion. Anchorage-independent growth was also decreased by CD44s re-expression as a separate protein (**Figure 5b**).

3.4 Cell adhesion assay

In PC-3M cells, those expressing CD44s as a separate protein had increased adhesion to hyaluronan [and osteopontin?] compared to untreated cells or CD44s-fusion cells. In the Dunning CD44s-separate cells, significant differences in ligand affinity were not noted (**Figure 6**).

3.5 Docetaxel sensitivity assay

Compared to untreated PC-3M cells or those expressing luciferase alone, cells with re-expression of CD44s as a fusion (or separate?) protein demonstrated increased sensitivity to Docetaxel doses of 0-50 nM (**Figure 7**). The Dunning cells proved to be insensitive to Docetaxel at all doses including 100 nM (data not shown) and were not further studied.

4. Discussion

In the PC-3M cells, re-expression of CD44s as shown by strong signal when luciferin substrate was added to the flask, and by western blot analysis, was successful. As expected [11](Gao, 1998), there was increased hyaluronan binding in the transfectants. Growth and invasion assays….Increased invasion with fusion?

In the Dunning cells, our inability to document re-expressed CD44s at the protein level called into question the specificity of results from studies of that cell line. The lack of increased binding of the transfectants to hyaluronan, unlike what was expected [11](Gao 1998), provided further evidence of lack

of a functional CD44s. In light of these observations, the altered invasion, growth, and anchorage-independent colony formation resulting from re-expression of CD44s as a fusion or separate protein must be regarded as non-specific. Enforcement of RNA interference against CD44v7-10 also appears to have been ineffective, likely because the sequence used differed by 4 bases from that of the rat [18](Hoffmann, 1991).

Merlin:

Merlin is a molecule whose conformation changes to a circularized, inactive form that inactivates its function. It is activated by phosphorylation under growth-promoting conditions and dephosphorylated under growth-inhibiting conditions including CD44-hyaluronan binding [~~11~~13]. Horiguchi et al. [~~16~~19] found that silencing of CD44 dephosphorylates merlin in benign cells. Moreover, both merlin and phospho-merlin were strongly expressed in DU145 PCa cells, but were weak to absent in LNCaP, PC3, 22RV1, and LAPC-4. Studying only the DU145 cells in further detail, they found that silencing total CD44 had no effect on these high levels of merlin and phospho-merlin, although another CD44 binding partner, the ERM (ezrin-radixin-moesin) proteins, had markedly reduced phosphorylation. This implied constitutive merlin phosphorylation. In PC-3M cells, we also found that baseline merlin and phospho-merlin were low to absent. These findings were not influenced by re-expression of CD44s as a fusion or separate protein. However, phospho-merlin was increased in cells with CD44v7-10 RNAi silencing. Seems counterintuitive! If v7-10 RNAi is increasing standard, then re-expression of CD44s should have the same effect.…

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Figure Legends

Fig. 1. Lentivector constructs. CD44s re-expression was achieved as a fusion protein with luciferase and as a separate protein. CD44s in human prostate includes exons 1-5 and 16-18, with a small portion of 20, according to our sequencing (3).

Fig. 2. Immunoblot hybridized to anti-total CD44, with protein extracts from untreated PC-3M cells (NT), cells re-expressing CD44s as a Fusion protein or a Separate (Sep) protein, and three preparations with RNA interference against CD44v7-10. The expected 66-kD cleavage product is seen but in Fusion cells there also is a 120-kD protein fused to the 60-kD luciferase moiety. β-actin is a loading control.

Fig. 3. Invasion and migration assays

Fig. 4. Soft agar colony formation assays. a. Cells transformed by pTracer plasmid. B. Cells infected with virus.

Fig. 5. Adhesion assays

Fig. 6.

Fig. 7.